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DIPLOMARBEIT

Molecular and immunological characterization of melon (*Cucumis melo*) allergens

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List of abbreviations

AAAAI	American Academy of Allergy, Asthma and Immunology
Ab	Antibody
AC	Affinity Chromatography
Ag	Antigen
BCA	Bicinchoninic acid
BSA	Bovine Serum Albumine
CD	Circular dichroism
Da	Dalton
DBPCFC	Double blind placebo controlled food allergy
DTT	Dithiothreitol
EAACI	European Academy of Allergy and Clinical Immunology
ELISA	Enzyme Linked Immunosorbent Assay
GI	Gastrointestinal Tract
HRP	Horseradish Peroxidase
IEF	Isoelectric Focusing
IEX	Ion Exchange Chromatography
Ig	Immunoglobulin
k	Kilo
L	Liter
M	Molar
mL	Milli Liter
nsLTP	Non-specific Lipid Transfer Protein
OAS	Oral Allergy Syndrome
OFC	Oral food challenge
PDS	Piel de Sapo
pI	Isoelectric Point
PR	Pathogenesis Related
SPT	Skin Prick Test
Th2 Cell	T Helper Cell Type 2
µL	Micro Liter

1 Objective

Food allergy is a health problem of great public concern. 2-4 % of the adult and 6-8 % of children suffer from food allergic symptoms. Accurate diagnosis of food allergy is the first step to improve the patient's quality of life.

The present diploma thesis was performed in the context of the European Union funded project, EuroPrevall (Prevalence, cost and basis of food allergy across Europe). EuroPrevall is a multidisciplinary integrated project involving clinicians, scientists, epidemiologists, and representatives of patient's associations and food industry. The project aims at investigating the prevalence and distribution of food allergies across Europe, determining the different patterns of food allergy, measuring the socio-economic impact of food allergy and developing new diagnostic tools providing a better correlation of *in vitro* diagnostic results with the clinical situation. Within EuroPrevall a library of purified and well-characterized food allergens will be established. So far, allergens from 16 foods (apple, peach, hazelnut, peanut, celery, kiwifruit, walnut, wheat, mustard, sesame, soy, cow´s milk, hen's egg, goat's milk, fish, and shrimp) were characterized.

The aim of the present diploma thesis is to define the allergen spectrum of melon, *Cucumis melo*. So far, three melon allergens have been described: Cuc m 1 (Cucumisin), a 67 kDa subtilisin-like protease, Cuc m 2 (profilin) the major melon allergen, a 13 kDa actin binding protein, and Cuc m 3, a 17 kDa pathogenesis-related (PR) protein belonging to the PR-1 family.

In the context of this study, further objectives are the standardization of melon extraction protocols, the development of melon protein purification protocols, the characterization of melon allergens regarding molecular and immunological features and the investigation of possible cross-reactive structures.

The panel of well-characterized allergens will be used to prove the concept of component resolved diagnosis and represents the basis for setting up novel diagnostics.

2 Background

2.1 Hypersensitivity

2.1.1 Definition

In 1906 the term “allergy” was defined by Clemens von Pirquet as an “altered capacity of the body to react to a foreign substance” (Pirquet, 1906). The actual definition of the European Academy of Allergy and Clinical Immunology (EAACI) is: “Hypersensitivity causes objectively reproducible symptoms or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects” (Johansson et al., 2001). Hypersensitivity can be divided into allergic hypersensitivity and non-allergic hypersensitivity. The EAACI position paper states that the term allergic hypersensitivity is appropriate when immunological mechanisms have been demonstrated and does not include classical responses to infections, autoimmunity, or toxic reactions. The term allergy can be used instead of allergic hypersensitivity. Classification of hypersensitivity is shown in Figure 1.

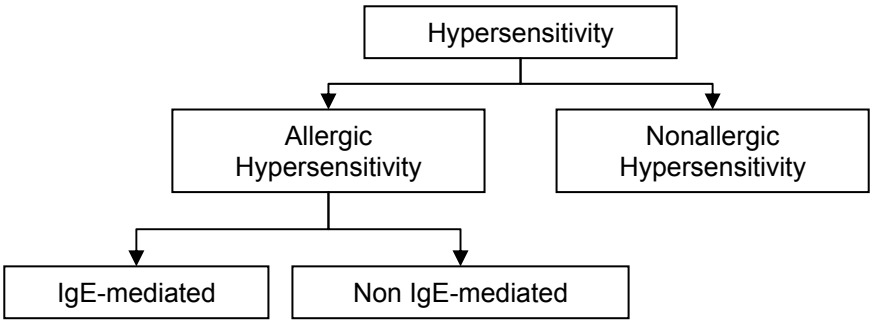


Figure 1 Classification of hypersensitivity. Figure according to Johansson et al. (Johansson, et al., 2001)

In 1968, Gell and Combs categorized the immunological mechanisms into four types. Type I is the immediate-type of hypersensitivity and the reaction is

mediated by IgE antibodies. IgG mediated type II hypersensitivity is also known as cytotoxic reaction. The antibodies are directed against endogenous antigens, like cell surface or matrix antigens. The type III reaction is mediated by IgG and IgM antibodies which are directed against soluble antigens. Type IV hypersensitivity is mediated by T-cells. The reaction is often directed against self antigens. So far, the classification is still generally accepted.

2.1.2 Food allergy

Food allergy is one of the most recognized diseases among allergies. So far, there is a lack of global agreement on definitions. Nowadays two definitions are accepted, one from the American Academy of Allergy, Asthma and Immunology (AAAAI) and one from the EAACI. Differences are shown in Figure 2.

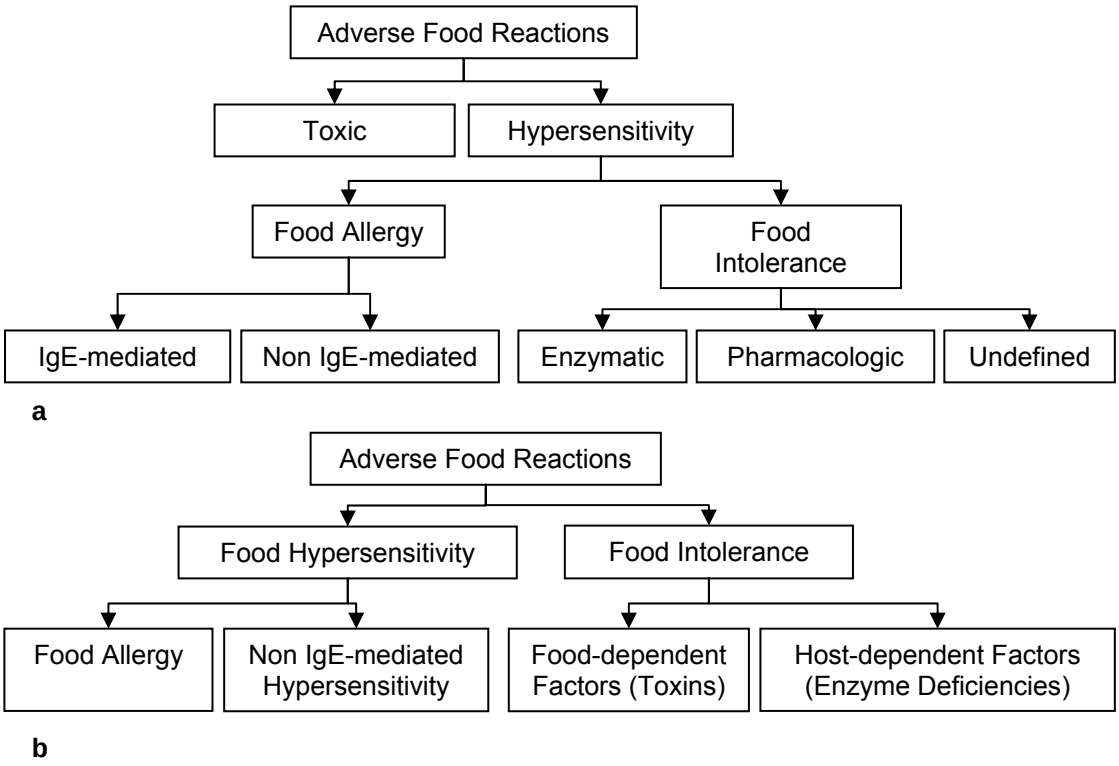


Figure 2 Classification of adverse food reactions (a) European classification, (b) American classification for food allergy. Figure according to Asero et al. (Asero et al., 2007)

According to the EAACI, food allergy is defined as an adverse immune response against non toxic food ingredients (Bruijnzeel-Koomen et al., 1995). By contrast, non immune-mediated reactions are due to enzymatic or physiological shortcomings. Furthermore, food allergy can either be IgE mediated (e.g. oral allergy syndrome) or non IgE mediated (e.g. celiac disease) (Bruijnzeel-Koomen, et al., 1995).

The best type of food allergy studied so far is the immediate type I reaction. Other immune-mediated mechanisms involved in food allergy may play a role but the knowledge is comparatively poor. The mechanism of IgE mediated allergy can be dissected into sensitization and effector phase.

2.1.2.1 IgE mediated immune mechanism

Primary exposure to an allergen in an atopic individual results in sensitizing mast cells. Antigens are taken up by the antigen presenting cells (APC), which process the antigen and present it to T cells. Naïve T-cells bind to the APC and under influence of certain cytokine patterns naïve T cells differentiate to T helper cells type 2 (Th2 cell). Subsequently interleukin 4 and interleukin 13 secreted by Th2 cells stimulate antigen specific B lymphocytes specific to switch to IgE producing plasma B cells. The reason for developing a Th2 biased immune response is unknown, but the propensity towards Th2 development has a strong genetic basis (Janeway, 2005).

The resulting IgE antibodies bind to high-affinity Fc receptors on mast cells, called FcεRI. In allergic individuals, mast cells are coated by antigen specific IgE antibodies (Janeway, 2005).

The second encounter to the same allergen leads to an activation of sensitized mast cells and mediator release. Mast cells are activated by cross-linking of two allergen specific IgE antibodies via the allergen on their surface. Where the mast cells are activated depends on the route of the allergen entry. For instance, ingested allergens activate mast cells in the wall of the intestine, whereas inhaled allergens activate mast cells in the submucosal tissues of the bronchus. The cross-linkage results in a signal transduction in the mast cell and

leads to a release of preformed granula (degranulation), synthesis and secretion of lipid mediators, and cytokines (Bischoff and Sellege, 2003). The degranulation reaction is shown in Figure 3.

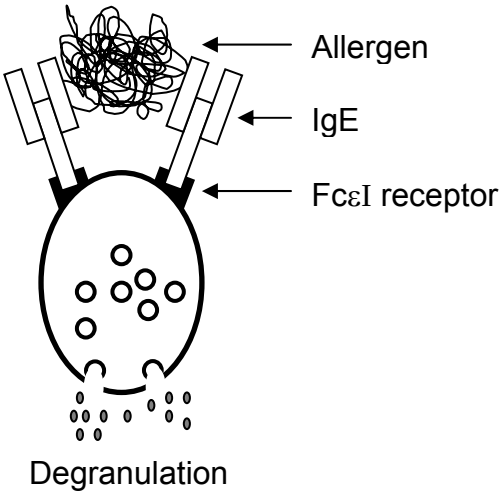


Figure 3 IgE cross-linking. Figure according to Janeway et al. (Janeway, 2005)

The most important mediator produced by mast cells is the vasoactive histamine. The produced cytokines stimulate the recruitment of leucocytes (eosinophils, neutrophils, Th2 cells). Eosinophils and neutrophils cause tissue damage, and Th2 cells trigger the reaction by producing more cytokines (Bischoff and Sellege, 2003).

The development of food allergy is multi-factorial and depends on genetic polymorphism, environmental conditions, mucosal barrier function, mucosal immune function, type and dosage of the food allergen, the route of allergen administration, and age of the afflicted individual (Bischoff and Sellege, 2003).
A key issue for understanding the pathophysiology of food allergy lies in the route of sensitization. Primary immunological recognition takes place in the gastrointestinal (GI) tract. The healthy GI immune system is able to tolerate harmless dietary antigens and commensal bacteria. Simultaneously it identifies and protects against harmful pathogens. However, the protective immune system can react against common, innocuous antigens. The failure to tolerate

commensal bacteria and food antigens predisposes a person to develop allergies (Bischoff and Sellege, 2003).

2.1.2.2 Food allergic symptoms

Food allergy causes a number of clinical conditions involving the skin, the gastrointestinal tract, or the airways. Symptoms of food allergic disorders range from mild local reactions to generalized severe life threatening symptoms, like anaphylaxis. Frequently, the severity of symptoms depends on the eliciting allergen (Sicherer, 2002). A summary of allergic reactions are shown in Table 1.

	IgE-mediated	Non-IgE mediated
Skin	Urticaria	Atopic dermatitis (subset)
	Atopic dermatitis	
Gastrointestinal	Oral allergy syndrome	Enterocolitis syndrome
	Gut anaphylaxis	Proctocolitis
	Eosinophilic gastroenteritis	Eosinophilic gastroenteritis
		Celiac disease
		Reflux (subset)
Respiratory	Asthma	
	Rhinitis	

Table 1 Examples of IgE- and non-IgE-mediated food allergic diseases. Table according to Sicherer (Sicherer, 1999)

Gastrointestinal reactions

The oral allergy syndrome (OAS) is mostly found in adults. OAS is a type of IgE mediated contact urticaria. It is characterized by local symptoms including itching and swelling of lips, tongue, palate, throat, ears, and nose within few minutes after eating eliciting foods (Sicherer, 2002).

OAS is often seen in patients allergic to pollen after eating fresh fruits and vegetables. The association is based on the cross-reactivity between homologous proteins in pollen and vegetable foods. Therefore, the classical

OAS is generally associated with pollen-related food allergy and also called pollen-food allergy syndrome (Asero, et al., 2007).

Chronic allergic disorders affecting the gastrointestinal (GI) tract are: protein enterocolitis, protein-induced protocolitis, protein enteropathy and celiac disease. IgE-mediatied symptoms in the gastrointestinal tract range from nausea, vomiting, and abdominal pain to diarrhoea. Infants are mainly affected and the common eliciting foods are cow's milk and soy (Sicherer, 2002).

Skin reactions

Foods often induce skin disorders in allergic patients. The most frequent skin reaction in adult, IgE-mediated food allergic patients is acute urticaria. Symptoms develop within minutes after ingestion of the offending food. A further skin symptom is contact urticaria. The handling of raw foods, like fish, fruits and vegetables causes skin reactions. The skin is also involved in subacute and chronic food allergic disorders. Atopic dermatitis is a chronically relapsing pruritic skin disease. It is common in childhood and rarely in adult allergic patients. Food allergy-related skin disease is primarily mediated by IgE antibodies (Sicherer, 2002).

Respiratory reactions

Respiratory symptoms in food allergic patients are not a single disorder and not very frequent. Usually, children are affected by asthma and rhinitis, but in some cases allergic asthma also occurs in adults. Particularly, allergic asthma occurs in workers exposed to the offending food, e.g. baker. Inhalation of certain foods or the steam from cooking foods, e.g. flour, green coffee, castor bean, soybean, spices, egg white, and crustacean may provoke allergic asthma in food sensitized patients (Sicherer, 2002).

Anaphylaxis

The severest allergic reaction of food is anaphylaxis. It is defined as "severe, life-threatening, generalized or systematic hypersensitivity reaction" (Johansson, et al., 2001). After a few minutes of food ingestion the patients may

develop pruritus, urticaria, angioedema, laryngeal edema, bronchospasm, abdominal cramps, vomiting, diarrhoea, cardic arrhythmia, hypotension, and shock. Food allergens are a common cause of anaphylaxis, foods most commonly involved are peanut, tree nuts, fresh fruits, celery, seeds, legumes, seafood, egg and milk. A special form of anaphylaxis is the exercise-induced anaphylaxis. Food intake and exercises in the 2-4 hours following the ingestion induces generalised reactions to the food (Fernández-Rivas and Miles, 2004)

2.1.2.3 *In vivo* tests for diagnosis of food allergy

The skin prick test (SPT) is the most frequently used *in vivo* test for diagnosis of food allergy. Food allergen extracts are applied into the epidermis using a lancet needle. Skin testing with native foods is called prick to prick test. In this test, the lancet needle is plunged several times into the food immediately before pricking the patient's skin. The allergenic test protein interacts with food-specific IgE. Skin tests are judged positive if the wheal diameter is at least 3 mm and the ratio of the wheal is at least 0.25 times the wheal of histamine control (positive control; sodium chloride as negative control) (Poulsen et al., 1993). A negative skin test with food extracts represents a good method to rule out an IgE-mediated food allergy, whereas a positive test does not necessarily predict clinical reactivity. At present, the gold standard of diagnosis is the controlled oral food challenges (OFC). OFC are performed by feeding the patient the suspected food under in a clinical setting. The challenges can be done open, single-blinded, or double-blinded. The double-blind placebo-controlled food challenge (DBPCFC) is considered the gold standard for the final diagnosis of food allergy. To date, this test is not routinely used, because the test is time consuming and expensive (Asero, et al., 2007).

Component-resolved skin test diagnosis is a novel form of *in vivo* skin diagnosis. Component-resolved diagnosis (CRD) is based on pure allergen molecules which are either produced by recombinant expression or by purification from natural source. It enables a better definition of clinical reactivity and provides evidence for patient tailored immunotherapy (Valenta et al., 2007).

2.1.2.4 Prevalence

Food allergy is a health problem of public concern. In the last years the understanding of food allergies has increased, but the uncertainty regarding the prevalence of food allergy is still there. Food allergy is believed to affect 1.5-2.5 % of adults (Young et al., 1994) and 6-8 % of children (Bock, 1987). The uncertainty regarding the prevalence of food allergy is due to different factors. Recently, Zuidmeer et al. have highlighted some of these factors. One problem is overestimation. A majority of studies about prevalence are based on self reported reactions to food. The prevalence of food allergy based on self-reported assessments is often well above compared to those based on objective assessment that is clear cut diagnosis. In the Dutch adult population, self-reported food allergy and intolerance was found to be 12.4 %, whereas truly diagnosed food allergy based on DBPCFC was found in 2.4 % (Jansen et al., 1994).

Ignorance about botanical categorization of foods (peanut referred to legume) of the public leads to an overestimation of the prevalence of nuts allergy. Additionally certain fruits could stimulate the tissue mast cells directly, causing symptoms that are similar to food allergic reactions, and can be misinterpreted for allergic reactions. Also spices could mimic food allergic reactions. In summary, the number of people suffering from adverse reactions to food is much higher than the number of food allergic patients (Zuidmeer et al., 2008).

The choice of the diagnostic procedure is also affecting the number of potential diagnosed patients. Positive skin prick tests to plant derived foods in pollen allergic patients could be the result of IgE cross-reactivity without symptoms. The prevalence of plant derived foods depends on presence of sensitizing pollen and rise or fall according to the pollen season (Zuidmeer, et al., 2008).

The high variability in prevalence of food allergy across the world may also be explained by differences in nutritional habits and food processing. So far, melon allergy has been reported in the Mediterranean region, where it is one of the frequently consumed fruits. Prevalence of peanut allergy may depend on the processing methods. In the USA roasted peanuts are mainly consumed and

peanut is one of the most important elicitor of food allergy (Sicherer et al., 2003), whereas in China boiled peanuts are mainly consumed and there, peanut allergy is rare (Beyer et al., 2001).

The prevalence data regarding gender differences in food allergy are inconsistent, especially those between questionnaire studies and detection of specific IgE antibodies or positive skin prick test. For example, Fernández-Rivas et al. have found significant differences in sex distribution within a group of 389 patients allergic to apple. In the Netherlands, Austria, and Spain, female apple allergic patients dominated, but not in Italy (Fernández-Rivas et al, 2006).

With reference to asthma and food allergy, the prevalence is higher in boys before puberty, while this gender ratio is reversed after puberty (Becklake and Kauffmann, 1999). Only in self-reported prevalence studies a gender difference exists with significantly more food allergic females than males (Chen et al., 2008). So far, no explanation has been proposed.

An increase of food allergy prevalence may be caused by importing exotic food in regions, where these foods were not regularly eaten before. Kiwifruit has been introduced onto the European food market in the 1970s. Subsequently, the first allergy to kiwifruit was reported in 1981 (Fine, 1981). Today, kiwifruit has become one of the major elicitor of plant food allergy in Europe (Mattila et al., 2003).

2.1.2.5 Food allergens

Allergens are defined as “an antigen causing allergic disease” (Johansson et al., 2004). Most food allergens reacting with IgE antibodies are non-toxic and harmless proteins deriving either from animal or from plants (Bruijnzeel-Koomen, et al., 1995). So what makes an antigen to be an allergen? It is important to understand the pathomechanism of allergy, in order to treat symptoms, and to assess new allergenic proteins. Although the question can not be completely answered yet, some general principles of allergens are already known.

Structural features responsible for allergenicity are solubility, stability, size, and an overall compact fold. In particular, allergens sensitizing via the GI tract retain their structural integrity during ingestion and they are taken up by the intestinal as an intact protein. Food allergens need to endure the conditions of the GI tract, because loosing their original allergenic three-dimensional structure is often accompanied by down regulating their allergenicity. Furthermore, allergens can also trigger allergic symptoms in an already sensitized subject (Aalberse, 2000). Depending on the characteristics, two forms of food allergens can be distinguished: class I and class II food allergens (Breiteneder and Ebner, 2000).

Class I allergens are proteins or glycoproteins, resistant to pepsin and stable to high temperature and extreme pH conditions. Due to these characteristics they reach the GI tract in almost unmodified form, and are still allergenic to sensitize via the gastrointestinal tract. Important members of Class 1 allergens have been identified from cow's milk, hen's egg, and legumes (Breiteneder and Ebner, 2000). These allergens are the major allergen sources for food allergy in infancy and early childhood (Sicherer, 2002).

In contrast, class II allergens are unstable during the digestion process, thermo labile and unable to sensitize. The primary sensitization occurs through the respiratory tract by inhalant allergens. Resulting specific IgE antibodies raised against the inhalant allergens also cross react with homologous allergens in fruits and vegetables. After ingestion of raw fruits or vegetables, patients allergic to pollens develop symptoms (Breiteneder and Ebner, 2000). For example, ragweed sensitive persons react to melon (Anderson et al., 1970). Typically cooked foods are tolerated.

2.1.2.6 Cross-reactivity

Cross-reactivity is defined as an immune response against the sensitizing antigen as well as towards structurally related antigen (Aalberse, 2000). It can be divided into IgE mediated and T-cell mediated cross-reactivity, respectively, linked with or without clinical symptoms. The reaction is determined by sharing

primary and tertiary structural features. Cross-reactivity between 2 or more allergens is likely in case of more than 50 % of sequence identity, whereas cross-reactivity is rare below 50 %. Additionally, cross-reacting allergens have a similar overall fold (Aalberse, 2000).

Cross-reactivity may exist between taxonomically related and non-related allergenic proteins. The conservation of certain proteins from taxonomically non-related species can cause cross-reactivity of allergens, for example in pollen and vegetable food (Aalberse, 2000).

Not every allergen specific IgE recognition of homologous allergens results in clinical symptoms. On one side, cross-reactivity is only recognized by in vitro serum tests. For example watermelon allergy has been reported to be linked to ragweed pollen allergy (Anderson, et al., 1970), but there is a lack of clinical reports confirming this finding (Vieths et al., 2002). On the other side between 50-93 % of birch pollen allergic patients show IgE-mediated reactions to pollen-related foods (Dreborg, 1988). Some of the important cross-reactive structures are: Bet v 1 homologues, profilins, cross-reactive carbohydrate determinants, non-specific lipid transfer proteins (nsLTP), thaumatin-like proteins (TLP), and calcium-binding proteins (CBP) (Vieths, et al., 2002).

2.2 Melon allergy

Muskmelon belongs to the *Cucurbitaceae* family as well as cucumbers, squashes (including pumpkins), and watermelons. Melon is a false berry that means it is equipped with an inferior ovary. The fruit is cultivated on the ground in warmer regions and it takes between 3 and 4 months to mature.

Piel de Sapo melon is a cultivar of the *Inodorus* group. The dark green skin, that shows yellow lines when ripe, is responsible for the name. The Spanish name Piel de Sapo means toad skin melon. Piel de Sapo melon fruits are oval and weigh around 3 kg at maturity. The flesh colour range from yellow near the center to pale green near the rind, it is shown in Figure 4.

Piel de Sapo melon was developed in Spain and now this kind of melon presents one of the most common consumed cultivars in Spain. In Austria, Piel de Sapo melon is less common, but the market is increasing. Since years, this melon cultivar has been sold in the Austrian supermarkets. Before, it was only available at special fruit markets.

Figure 4 Piel de Sapo melon

Melon is a relevant elicitor of food allergy. In Europe, melon allergy has been reported for Southern Europe (Cuesta-Herranz et al., 2000, Garcia Ortiz et al., 1995, Tavares et al., 2008). Furthermore, melon is a cause of food allergy in the USA (Anderson, et al., 1970) and Japan (Inomata et al., 2007).

Allergic symptoms to melon range from mild to severe life threatening reactions. The most common symptoms are the oral allergy syndrome in context of pollinosis (Rodriguez et al., 2000). In some cases melon causes generalized urticaria, respiratory difficulty, and hypotension (Figueredo et al., 2003). Therefore melon is a potential cause of life threatening reactions. Recently, a case of contact hypersensitivity to melon was described (Garcia et al., 2004).

Rodriguez et al. have been reported that isolated melon allergy is rare. Most patients either suffer from allergic rhinitis, asthma or both to different pollens. 19 melon allergic patients were diagnosed by DBPCFC. 18 of these were also sensitized to pollens from grass, tree, or weed. Additionally, melon allergic persons showed allergic reactions to various fruits. The most common fruits were avocado, banana, kiwifruit, watermelon and peach (Rodriguez, et al., 2000).

In most cases the patients develop allergy to pollen first, followed by symptoms after consumption of melon. After the *Rosaceae* family, melon is the main cause of eliciting a positive skin test response in pollen sensitized patients in Spain (Cuesta-Herranz, et al., 2000). The results of the group around Florido Lopez are in agreement with those of Cuesta-Herranz et al. The second fruit frequently causing OAS in *Olea europaea* sensitized persons is melon. Results are different in pollen sensitized patients suffering from generalized, systemic reactions to fruits. In this patient's group peach (84.21 %), nut (73.68 %), and kiwifruit (52.63 %) are the common elicitors of positive skin prick tests (Florido Lopez et al., 2002).

2.2.1 Melon allergens

According to the "Official list of allergens" of the International Union of Immunological Societies (IUIS) only allergens from the species *Cucumis melo* are listed in the plant order *Cucurbitales*. So far, three melon allergens have been identified. Cuc m 1, a subtilisin like protease, Cuc m 2, the melon profilin, and Cuc m 3, a member of the PR 1 familiy.

2.2.1.1 Cuc m 1

Cucumisin, Cuc m 1, belongs to the subtilisin like serin protease superfamily, termed subtilases. The subtilisin like serin protease superfamily can be divided into six subgroups: subtilisin, thermitase, proteinase K, lantibiotic peptidases,

kexin and pyrolysin. All members of the protein superfamily are endoproteases or tripeptidylpeptidases. Subtilases are distinguished from other serin proteases by a highly conserved catalytic triad: serine, aspartate and histidine. Serin acts as a nucleophile, aspartate as an electrophil, and histidine as a base. Subtilases occur in archea, bacteria, fungi, eukaryotes, and viruses. In higher eukaryotes the subtilases have developed from unspecific enzymes to highly specific enzymes. The fact that they have been identified in numerous organisms suggests that they are ubiquitous (Siezen and Leunissen, 1997).

The family subtilisin is the second largest serine protease family with over 200 members. According to the AllFam (www.meduniwien.ac.at/allergens/allfam) database seventeen allergens belong to the subtilisin-like serine proteases family. Most allergens from this family are fungal allergens, except Cuc m 1, the only plant food allergen and Bac 1 Subtilisin, the only bacteria allergen (AllFam: www.meduniwien.ac.at/allergens/allfam). The allergenic potential of these proteases is well understood. The biochemical mechanism to migrate into the organism was investigated of the major dust mite allergen Der p 1, a cysteine protease. The protease cleaves the tight junction protein occludin, hence increasing the epithelial permeability and facilitating its entry into the tissue. Additionally, Der p 1 acts directly on cells of the human immune system by cleaving cell-surface proteins and dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin (Radauer et al., 2008).

Cucumisin is a member of the subtilisin family based on sequence similarities. Cucumisin is the first known allergenic plant protease from this family. It was isolated in 1975 from melon juice (Kaneda and Tominaga, 1975). In 1994, the sequence of Cuc m 1 was determined (Yamagata et al., 1994). Cucumisin is an enzyme of 67 kDa showing weak autolytic activity. The 67 kDa protein resolves into a 54 kDa protease and a 14 kDa polypeptide. The autolyzed 54 kDa protease is stable. In melon fruits, Cucumisin accounts for 10 % of the total protein (Yamagata, et al., 1994).

Cuesta-Herranz et al. have identified Cucumisin as an allergen of melon fruit (Cuesta-Herranz et al., 2003). The authors showed that proteins of 67 kDa, 54 kDa, 49 kDa, 36 kDa, and 14 kDa shared the same N-terminal sequence:

TTRSWDFL. Sequence alignment identified this as the N-terminal sequence of Cucumisin. The lower molecular weight proteins are identified as fragments of Cuc m 1. Among those, the 36 kDa fragment is the most frequently recognized allergen by IgE from allergic patient's sera. This fragment could represent the dominant B-cell epitope of Cucumisin. Furthermore, Cuesta-Herranz et al. have suggested, that Cucumisin could play a role as a new pan-allergen in plant foods, because of its occurrence in other plant species, e.g. tomato, soybean, rice, barley, or latex (Cuesta-Herranz, et al., 2003).

Sequence:

```
1                                                                    60
MSSSLIFKLF FFSLFFSNRL ASRLDSDDDG KNIYIVYMGR KLEDPDSAHL HHRAMLEQVV
61                                                                  120
GSTFAPESVL HTYKRSFNGF AVKLTEEEAE KIASMEGVVS VFLNEMNELH TTRSWDFLGF
121                                                                 180
PLTVPRRSQV ESNIVVGVLD TGIWPESPSF DDEGFSPPPP KWKGTCETSN NFRCNRKIIG
181                                                                 240
ARSYHIGRPI SPGDVNGPRD TNGHGTHTAS TAAGGLVSQA NLYGLGLGTA RGGVPLARIA
241                                                                 300
AYKVCWNDGC SDTDILAAYD DAIADGVDII SLSVGGANPR HYFVDAIAIG SFHAVERGIL
301                                                                 360
TSNSAGNGGP NFFTTASLSP WLLSVAASTM DRKFVTQVQI GNGQSFQGVS INTFDNQYYP
361                                                                 420
LVSGRDIPNT GFDKSTSRFC TDKSVNPNLL KGKIVVCEAS FGPHEFFKSL DGAAGVLMTS
421                                                                 480
NTRDYADSYP LPSSVLDPND LLATLRYIYS IRSPGATIFK STTILNASAP VVVSFSSRGP
481                                                                 540
NRATKDVIKP DISGPGVEIL AAWPSVAPVG GIRRNTLFNI ISGTSMSCPH ITGIATYVKT
540                                                                 600
YNPTWSPAAI KSALMTTASP MNARFNPQAE FAYGSGHVNP LKAVRPGLVY DANESDYKFL
600                                                                 660
CGQGYNTQAV RRITGDYSAC TSGNTGRVWD LNYPSFGLSV SPSQTFNQYF NRTLTSVAPQ
661                                                                 720
ASTYRAMISA PQGLTISVNP NVLSFNGLGD RKSFTLTVRG SIKGFVVSAS LVWSDGVHYV
721      731
RSPITITSLV T
```

Length: 731 amino acids Molecular Mass: 78820 Da pI: 8.41

Figure 5 Molecular data of Cuc m 1; Uniprot Number Q39547

2.2.1.2 Cuc m 2

Cuc m 2 is a member of the profilin family. Profilins are actin-binding, low-molecular-weight proteins. The biochemical function of profilin is the regulation of the actin cytoskeleton. Profilins have been well preserved during evolution. Profilins from mammals, lower eukaryotes, and plants share an identical fold. Despite their identical function and the high sequence identity, the amino acid sequence differs among organisms from different taxonomical orders. However, among plant profilins sequences similarities are quite high, at least 75 % (Radauer and Hoffmann-Sommergruber, 2004).

Profilins are found in all eukaryotic cells. They have been identified as allergens in many fruits and vegetables as well as in pollens of grasses, weeds and trees. Profilins from pollens and plant foods are highly cross-reactive. Profilin-sensitized patients show allergic symptoms towards a large number of botanically unrelated plants. Asero et al. observed that more than 50 % (34 of 60 patients, 57%) of profilin-sensitized patients showed clinically relevant cross-sensitization to plant-derived foods (Asero et al., 2008). Thus, profilin should be considered as a clinically relevant food allergen for certain species. The pan-allergen profilin may be responsible for several pollen-food allergy syndromes (Egger et al., 2006). However, whether the presence of profilin specific IgE is of clinical relevance for all species is not yet agreed (Wensing et al., 2002).

Profilins are quite heat stable. In celery, conventional cooking in water for 20 minutes did not affect the allergenicity, whereas heating to 100°C for 30 minutes reduced almost the whole IgE binding capacity (Jankiewicz et al., 1996). Food processing treatments such as gamma-irradiation, drying, powdering, ultra high pressure treatment, or high voltage impulse treatment did not affect the allergenicity (Jankiewicz et al., 1997). In contrast, profilins are labile to gastric digestion as shown for apple profilin (Ma et al., 2006).

Profilins were first described in 1977 by Carlsson et al. (Carlsson et al., 1977). In 1991 profilin was reported as a minor allergen in birch pollen (Valenta et al., 1991). It was the start for further intensive research for the allergenic activity of profilins. In nearly every type of plant allergen sources such as pollens, fruits, vegetables, spices, seeds and latex, profilins were identified as allergens. The

melon profilin was first described by Rodriguez-Perez et al. (Rodriguez-Perez et al., 2003). Two years later, López-Torrejón et al. isolated and identified the 13 kDa protein as profilin. Profilin is the major allergen in melon extract, almost all melon-allergic sera display melon profilin specific IgE (Rodriguez-Perez, et al., 2003) (Lopez-Torrejon et al., 2005).

The total amino acid sequence is already known. The melon profilin sequence is 131 amino acids long (Sankian et al., 2005) and shown in Figure 5. The calculated molecular mass of melon profilin is 14048 Da with a theoretical isoelectric point of 4.58. The sequence for Cuc m 2 was very close to profilins from fruits, such as peach Pru p 4 (98 % identity with Hev b 8), latex (84 % identity), and pollens (74 % and 71 % with Bet v 2 and ragweed, respectively) (Lopez-Torrejon, et al., 2005). So far, two major and two minor IgE epitopes have been identified in melon profilin (Lopez-Torrejon et al., 2007).

Sequence:
1 60 MSWQVYVDEH LMCEIEGNHL TSAAIIGQDG SVWAQSQNFP QLKPEEVAGI VGDFADPGTL 61 120 APTGLYIGGT KYMVIQGEPG AVIRGKKGPG GATVKKTGMA LVIGIYDEPM TPGQCNMIVE 121 131 RLGDYLIDQG L
Length: 131 amino acids Molecular Mass: 13934 Da pI: 4.58

Figure 6 Molecular data of Cuc m 2; UniProt Number: Q5FX67

To evaluate the stability of melon profilin, the recombinant (r) Cuc m 2 was subjected to simulated gastric fluid (SGF) and heat treatment. Recombinant Cuc m 2 was extensively digested in a SGF assay for 30 minutes. The allergen completely lost its capacity to bind IgE from melon-allergic patients and anti-profilin antibodies. Heat treatment had no effect on the IgE-binding to rCuc m 2 (Lopez-Torrejon, et al., 2005).

Lopez-Torrejon et al. found differences between natural and recombinant melon profilin. Recombinant Cuc m 2 was only detected by 18 of 23 (78 %) melon-

allergic sera. In contrast, all patients' sera recognized the natural allergen. Thus, the natural proteins seems to be a better diagnostic tool than the recombinant allergen (Lopez-Torrejon, et al., 2005). Furthermore, melon is a marker for profilin sensitization in patients sensitized to Bet v 1, Bet v 2, or both. Asero et al. showed a strong association between profilin sensitization and clinical allergy to melon, watermelon, citrus fruits, tomato, and banana. All these plant derived food have been rarely reported to elicit allergic reactions in non-profilin sensitized patients (Asero et al., 2003).

2.2.1.3 Cuc m 3

Cuc m 3 belongs to the pathogenesis related (PR) protein family 1. PRs are defined as proteins that are induced by pathogen attack, wounding or other physical or chemical stress. Furthermore, some proteins are inducible by the signalling compounds such as salicylic acid, jasmonic acid, or ethylene. The PR proteins can be grouped into 17 protein families, as shown in Table 2.

The pathogenesis related proteins are evolutionarily conserved families, individually differing widely in occurrence and, where known, activity (van Loon, et al., 2006). The common biological properties are low molecular weight, stability at low pH, and resistance to proteases. 25 % of the known plant allergens belong to one of the pathogenesis related protein groups. Plant derived allergens have been identified in 8 PR families: PR1-PR 5, PR 8, PR 10, and PR 14 (Hoffmann-Sommergruber, 2002) (Asensio et al., 2004).

The PR 1 family members code for 135 amino residues and have a molecular weight of 14-17 kDa. Six conserved cysteine residues form disulfide bridges, which lead to a compact 3-dimensional structure. PR 1 proteins are highly conserved, 35 % sequence identity was found in all PR 1 proteins. PR 1 proteins have been identified in almost all known plant species, in fungi, insects, and vertebrates. The biological function of PR 1 proteins is not known. Some members have been associated with activity against oomycetes (van Loon, et al., 2006).

Family	MW (kDa)	Properties
PR 1	15-17	unknown
PR 2	25-35	β-1,3-glucanase
PR 3	25-35	Chitinase type I, II, IV, V, VI, VII
PR 4	13-19	Chitinase type I, II
PR 5	22-24	Thaumatin-like
PR 6	6	Proteinase-inhibitor
PR 7	69	Endoproteinase
PR 8	28	Chitinase type III
PR 9	39-40	Peroxidase
PR 10	17-18	Ribonuclease-like
PR 11	41-43	Chitinase, type I
PR 12	5	Defensin
PR 13	14	Thionine
PR 14	7-12	Lipid-transfer protein
PR 15	26	Oxalate oxidase
PR 16	22	Oxalate-oxidase-like
PR 17	24-26	unknown

Table 2 PR protein family members. Table according to van Loon et al. (van Loon et al., 2006)

The melon PR 1 protein, Cuc m 3, is of 16097 Da, has a blocked N-terminus, and is highly resistant to several proteases. Sequence alignment has shown a strong similarity with PR 1 members from grape and cucumber. In melon fruit, Cuc m 3 allergen is a minor allergen.

a

Sequence:

```
1                                                                    60
MLPFSFAQDS IKDFVDAHNA ARAQVGVGPV HWNKTVADYA HQYANKRIKD CNLVHSKGPY
61                                                                  120
GENIAWGSRN LAGTVAVRMW VSEKQFYNYD TNSCVRGKMC GHYTQVVWRN SVRIGCAKVR
121                             151
CKSGGTFITC NYDPRGNIRG QRPYGEGTLQ L
```

Length: 151 amino acids Molecular Mass: 19097 Da pI: 9.53

Figure 7 Molecular data of Cuc m 3; NCBI Protein Database Number: ACB45874

Only 14 % of tested sera of melon allergic patients have shown positive skin prick test responses to Cuc m 3 (Asensio, et al., 2004). The amount of Cuc m 3 in the fruit can vary during growth. Cuc m 3 is up regulated upon pathogen attack. Cuc m 3 is the first example of a PR 1 allergen involved in food allergy (Asensio, et al., 2004).

2.2.2 Cross-reactivity of melon allergens

The first description of allergic reactions to melon was in 1970 by Anderson et al. in association with ragweed pollinosis (Anderson, et al., 1970). Subsequently, several studies reported the link between hypersensitivity to melon and allergy to different pollens. As mentioned above, the first described syndrome was called ragweed-melon-banana (Anderson, et al., 1970). Afterwards, further cross-reactive reactions to melon were published. García Oritz et al. showed by immunoblot analysis that melon and *Plantago* pollen as well as the grass *Dactylis glomerata* share common allergens. However clinical data are still lacking (Garcia Ortiz et al., 1998). Further cross-reactivity was shown between melon and *Olea europea* (Florido Lopez, et al., 2002). Melon also shares IgE-binding proteins with other members of the *Cucurbitaceae* family, e.g. pumpkin, watermelon, cucumber, and zucchini (Reindl et al., 2000). A taxonomic cross-reactivity is likely between the members of the plant family.
Melon is an allergy eliciting fruit in the latex-fruit syndrome (Brehler et al., 1997) and additionally the most associated fruit with pollinosis in Spain (Garcia Ortiz, et al., 1998). The cross-reacting allergen has not yet been reported.
Profilin is one of the possible cross-reactive allergens. The panallergen exists in latex (Hev b 8) (Ganglberger et al., 2001) and in melon (Cuc m 2) (Lopez-Torrejon, et al., 2005). Maybe profilin sensitization could explain the cross-reaction between melon and latex. Cuesta-Herranz et al. have been suggested that Cucumisin is the responsible cross-reacting allergen between melon and latex (Cuesta-Herranz, et al., 2003). In the *Olea europaea* pollen-melon syndrome, Ole 7, a pollen nsLTP seems to be responsible for the cross-

reactivity in patients with systemic reactions (Florido Lopez, et al., 2002). However, to date no melon lipid transfer protein has been identified.

3 Materials and Methods

3.1 Materials

Chemicals	Suppliers
Mouse anti-human IgE antibodies	BD Pharmingen, Franklin Lakes, NJ, USA
Tris-(hydroxymethyl) aminomethane Coomassie Brilliant Blue R-250 (CBB) Dithiothreitol (DTT) 3-(Cyclohexylamine)-propyl sulfonic acid (CAPS) Glycin p.A. Aminoacetic acid 4-Nitro blue tetrasodium chloride (NBTC) 5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS)	Biomol GmbH, Hamburg, Deutschland
N,N'-Methyl-bis-acrylamide	Biorad, Hercules, USA
Swine anti-rabbit IgG AP-conjugated	Dako, Glostrup, Denmark
Page Ruler Prestained Protein Ladder	Fermentas
Methyl-α-D- Mannosepyranosid	Fluka Chemie GmbH, Buchs, Schweiz
Con A Sepharose DEAE Sepharose Fast Flow SP Sepharose Fast Flow IPG Buffer	GE Healthcare, Little Chalfont, UK
Rabbit anti-mouse IgG + IgM AP-conjugated	Jackson ImmunoResearch, Est Grove, PA, USA
Ammonium Persulfate	Life Technologies, Gaithersburg, USA
Acetone $CaCl_2$ Methanol (MeOH) Ethanol (EtOH)	Merck, KGaA, Darmstadt, Deutschland

NaCl NaH_2PO_4 x 2 H_2O Na_2HPO_4 x 12 H_2O NaN_3 CH_3COOH (HAc)	
Complete EDTA-free Protease inhibitor cocktail tablets	Roche Diagnostics GmbH, Mannheim, Deutschland
Albumin Fraction V Arcylamide Bromphenol blue sodium-salt Glycerine Milk powder Sodium dodecyl sulfate (SDS) ultra pure N,N,N,N-tetramethylethylenediamine (Temed)	Roth, Karlsruhe, Deutschland
FAST p-Nitrophenyl Phosphate Tablet set Polyvinylpolypyrolidone (PVPP) Sodiumdiethyldithiocarbamattrihydrat (DIECA) Tween 20	Sigma Aldrich Chemie GmbH, Steinheim, Deutschland

Table 3 Materials

3.2 Methods

3.2.1 Melon extract

3.2.1.1 Melon extract preparation

Extraction means separation of soluble and insoluble compounds of a mixture by dissolving soluble substances in a suitable solvent. In this case the target components are the proteins which should be totally dissolved in the extraction buffer.

Solutions

Sodium Phosphate Buffer:

Sodium Phosphate buffer of a given pH is composed of a monobasic and dibasic sodium stock solution.

A: 1 M solution of monobasic sodium phosphate (Na_2HPO_4)

B: 1 M solution of dibasic sodium phosphate (NaH_2PO_4)

10 mM sodium phosphate buffer, pH 7

57.7 mL 1M Na_2HPO_4

42.3 mL 1M NaH_2PO_4

Diluted to a total of 1 L with Aqua dest

Extractions buffer

10 mM sodium phosphate buffer, pH 7 containing

10 mM DIECA

3 mM NaN_3

2 mM EDTA

20 g/L PVPP

10 µL/L DTT

Procedure

Melon extract was prepared from fresh material. Five hundred grams of Piel de Sapo melon (*Cucumis melo var. Inodorus*) were peeled, the pulp cut into small pieces, and freeze-dried. The dried material was homogenized in 200 mL extraction buffer. After mixing the pH decreased to pH 6.6. The extract was recalibrated to pH 7.0 with 1M NaOH. Four protease inhibitor tablets were added and the extract was stirred at 4°C for one hour.

The suspension was clarified by centrifugation (25,000 g at 4°C for 1 hour), subsequently the supernatant and the pellet were further used.

- The pellet was resuspended in 0.5 M NaCl and centrifuged for 45 minutes (35,000 g at 4°C). After centrifugation the obtained supernatant was collected.
- The supernatant was freez-dried and the dried material was resuspended in Aqua dest. The suspension was $CaCl_2$ precipitated. The precipitation was carried out by adding salt to the protein extract up to 50 mM. The precipitate was dialyzed against 10 mM sodium phosphate buffer pH 7 for 24 hours (Molecular porous Dialysis Membrane, MWCO 1,000, Spectrum, Houston, Texas; USA). After dialysis, the melon extract was centrifuged (25,000 g at 4°C for 30 minutes). The supernatant was collected.

3.2.2 Purification of melon allergens

3.2.2.1 Protein purification by chromatography

Chromatography is one method to separate biomolecules according to differences in their physicochemical properties, e.g. charge, size, hydrophobicity, and biorecognition. The following purification steps were performed at “ÄKTA FPLC” (Fast-performance liquid chromatography, GE Healthcare, Little Chalfont, UK) using the software UNICORN (GE Healthcare, Little Chalfont, UK).

3.2.2.1.1 Affinity chromatography (AC)

AC separates proteins according to a reversible interaction between protein and a specific ligand bound to the solid phase of the chromatography medium. The target protein binds to the ligand while unbound proteins are washed away. The bound protein is recovered by changing the conditions, e.g. pH, ionic strength or polarity. Proteins are concentrated during binding to the solid phase and after elution they are collected in an enriched form.

3.2.2.1.1.1 Con A Sepharose column

Con A Sepharose is Concanavalin A coupled to Sepharose 4B and is routinely used for separation and purification of glycoproteins, polysaccharides and glycolipids. Concanavalin is a member of metalloproteins and contains two metal binding sites, Mn^{2+} and Ca^{2+}. The presence of both is essential to ensure an active Con A-metal complex and to preserve the binding activity of Con A molecules (GE Healthcare, 2001).

Solutions

Start buffer (buffer A)
20 mM Tris-HCl, pH 7.4
0.5 M NaCl

Elution buffer (buffer B)
Buffer A + 0.4 Methyl-α-D-Mannopyranosid

Procedure

The column was packed according to the handling instructions (GE Healthcare, Little Chalfont, UK) and was washed with at least 10 column volumes (CV) distilled water to remove the preservative. After connecting the column with the FPLC the column was equilibrated with buffer A. The buffers were filtered and degassed before applying to the column.

The melon protein extract was cleared by filtration (Rotilab-Spritzenfilter, 0.45 µm, PVDF, Carl Roth GmbH, Karlsruhe, Germany) and was applied on to the column. Bound proteins were eluted with buffer B gradient (0 to 100 %) within 10 CV at a flow rate of 1mL/min. 2 mL fractions were collected. The absorbance was measured at 280 nm.

The column was re-equilibrated with buffer A until the conductivity values were stable and was washed with milli Q water. For storage the column was kept in 20 % ethanol.

3.2.2.1.2 Ion exchange chromatography (IEX)

IEX is one of the most commonly performed techniques for purification of proteins, due to the high resolution and separation. IEX separates biomolecules according to differences in their net surface charge. The way of separation is based on variable degree of interaction with charged chromatography medium and differently charged proteins. Proteins net surface charge is highly pH dependent and will change as the pH of the environment. At a pH above its isoelectric point, a protein will interact with a positively charged medium or anion exchanger and, at a pH below its pI, a protein will bind to a negatively charged medium or cation exchanger (GE Healthcare, 2004).

3.2.2.1.2.1 DEAE Sepharose fast flow column (DEAE)

DEAE is a weak anion exchanger. The media contain diethylaminoethyl groups coupled to highly cross-linked 4 % agarose via chemically stable ether bonds. To enable a good interaction between target proteins and medium, the starting buffer must be at least one pH unit above the pI of the molecules (GE Healthcare, 2004).

Solution

Start buffer (buffer A)

20 mM Tris-HCl, pH 8.0

Elution buffer (buffer B)
Buffer A + NaCl

Procedure

The column was packed according to the handling instructions (DEAE Sepharose Fast Flow, GE Healthcare, Little Chalfont, UK) and was washed with at least 10 column volumes (CV) distilled water to remove the preservative. The column was equilibrated with 5 CV buffer A, 5 CV buffer B, and 5 CV buffer A. The next steps were performed as described in section 3.2.2.1.1.1. Briefly, melon proteins were loaded onto the column. The bound proteins were eluted with 0 to 100 % buffer B within 10 CV at a flow rate of 1mL/min. Fractions of 2 ml volume were collected.

3.2.2.1.2.2 Mono Q column

Mono Q is a strong anion exchanger. The functional group on the chromatographic medium is the quarternary amino group ($-CH_2-N^+(CH_3)_3$). The loading capacity is up to 20 mg protein per mL medium (GE Healthcare, 2004).

Solution

Start buffer (buffer A)
20 mM Tris-HCl, pH 8.0

Elution (buffer B)
Buffer A + 1 M NaCl

Procedure

Previously, the procedure was described in section 3.2.2.1.1.1. Melon protein extract was loaded onto a prepacked column (Mono Q 5/50 GL, GE Healthcare, Little Chalfont, UK). The binding proteins were eluted with 0 to 100 % buffer B within 20 CV at a flow rate of 1mL/min. Fractions of 1 ml volume were collected.

3.2.2.1.2.3 SP Sepharose

SP Sepharose (SP Sepharose Fast Flow, GE Healthcare, Little Chalfont, UK) is a strong cation exchanger. Functional groups of the media are Sulfopropyl groups.

Solution

Start buffer (buffer A)
10 mM Sodiumacetate, pH 5.4

Elution buffer (buffer B)
A + 1 M NaCl

Procedure

Previously, the procedure was described in section 3.2.2.1.1.1. The column was packed according to the handling instructions (GE Healthcare, Little Chalfont, UK). Melon protein extract was loaded onto the column. The binding proteins were eluted with 0 to 100 % buffer B within 20 CV at a flow rate of 1mL/min. 1 mL fractions were collected.

3.2.3 Molecular characterization of melon proteins

3.2.3.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

The SDS-PAGE was performed according to Laemmli (Laemmli, 1970).
SDS-PAGE is an electrophoretic method for separating proteins on the basis of molecular mass. The technique is performed in SDS containing polyacrylamide gels. The gels are attached to two buffer reservoirs. Then the proteins are loaded onto the gels and they migrate into the gel when an electric field is applied.
SDS denatures proteins and forms negatively charged proteins in proportion to the mass of proteins. The denaturation is important to form linear proteins to avoid different migration of proteins with the same molecular weights due to

differences in folding. DTT is a reducing agent. It is used to reduce disulfide linkages and to break up quaternary protein structures. Adding DTT is optional, when proteins are treated with DTT the SDS-PAGE is performed under reducing conditions. Bromophenol blue allows visualization of protein bands of the electrophoresis.

Solutions

Reagent C:
29.2 % Acrylamide
0.8 % Bis-Acrylamide
Dissolved in distilled water and filtered

Lower buffer
1.5 M Tris-HCl, pH 8.8
0.4 % SDS
Dissolved in distilled water

Upper buffer
0.5 M Tris-HCl, pH 6.8
0.4 % SDS
Dissolved in distilled water

Resolving gel 15 % (for 1 gel)

Reagent C	1.5 mL
Lower buffer	1.25 mL
Aqua dest	1.25 mL
Temed	2.5 µL
10 % APS	25 µL

Stacking gel 4.5 % (for 1 gel)

Reagent C	300 µL
Lower buffer	500 µL

Aqua dest	1.2 mL
Temed	1 µL
10 % APS	20 µL

4 x Sample Buffer
200 mM Tris-HCl, pH 6.8
300 mM DTT
4 % SDS
40 % Glycerine
0.04 % Bromphenol blue
Dissolved in distilled water

Electrophoresis buffer
25 mM Tris pure
192 mM Glycine
0.1 % SDS
Dissolved in distilled water

CCB-stain
1.125 % CBB R-250
50 % Methanol
10 % 100% HAc
dissolved in distilled water and filtered

Destainer
20 % Methanol
15 % 100% HAc
dissolved in distilled water

Procedure

Gels were prepared according to the handling instructions (Bio-Rad, Richmond, CA, USA). Gels need at least 45 minutes to polymerize and were stored at 4°C

until electrophoresis was performed. Samples were mixed with 4 x SB, heated for 5 minutes at 95°C, and centrifuged. Molecular weight marker and samples were applied to the gel slots and the electrophoresis was started (running condition: 160 Volt, 50 minutes, 1 x Electrophoresis buffer).
After the run Coomassie staining was used to visualize of proteins. Gels were stained for 30 minutes at room temperature (RT). Afterwards, the staining solution was discarded and the gel was rinsed with Aqua dest. Then, the gel was destained in destaining solution until the protein bands were detectable.

3.2.3.2 Determination of protein concentration

Determination of total protein content is based on the well known Biuret reaction, the reduction of Cu^{+2} to Cu^{+1} by protein under alkaline conditions. The BCA Protein Assay combines this reaction with the colorimetric detection of the cuprous cation (Cu^{+1}) using bicinchoninic acid (BCA). Two molecules of BCA bind one cuprous ion; this yellow-green complex shows a strong absorbance at 562 nm. The absorbance increases nearly linearly with increasing protein concentration. For determination of protein concentration a series of standards of proteins such as bovine serum albumin (BSA) is necessary (Pierce).

Solutions

BCA working reagent A (Thermo Scientific, Rockford, USA)
BCA working reagent B (Thermo Scientific, Rockford, USA)

Procedure

Preparation of Working Reagent (WR)
50 part reagent A and 1 part reagent B
200 µL WR per sample

Preparation of BSA standards:

Standard number	Volume BSA [µL]	Volume buffer [µL]	Final concentration [µg/mL]
0	0	160	0
1	4	156	50
2	8	152	100
3	12	148	150
4	8	72	200
5	10	70	250

Table 4 Preparation of BSA standards

Samples were diluted in buffer (buffer in which the samples were dissolved; e.g. 10 mM sodium phosphate buffer, pH 7). 25 µL of each standard or unknown sample was pipetted in replicate into the wells of a microtiterplate (Microplate, Greiner Bio-One, Greiner, Frikenhausen, Germany) and 200 µL WR was added to each well. The plate was covered (Foil EASYseal^{TM}, Greiner, Frikenhausen, Germany) and incubated at 37°C for 30 minutes. After cooling to RT, the absorbance at 562 nm was measured on the plate reader (SPECTRAmax, Molecular Devices GmbH, Munich, Germany) using SoftMax Pro Software.

3.2.3.3 Two-dimensional (2 D) electrophoresis

Two-dimensional gel electrophoresis is the combination of two high-resolution electrophoretic procedures: isoelectric focusing (IEF) and SDS-PAGE. The first dimension, the IEF, separates proteins according to their isoelectric point (pI). The pI is the specific pH at which the protein carries a zero net charge. At pH values below their pI, proteins are positively charged and at pH values above their pI, proteins are negatively charged. In a pH gradient, under the influence of an electric field, proteins migrate to the point, where its net charge is zero, thus proteins will be focused on their pIs. IEF is a very sensitive method, because it separates proteins according to very small charge differences. The second

dimension, SDS PAGE, separates proteins according to their molecular mass (Berkelman, 1998).

Urea and the zwitterionic detergent CHAPS denature and solubilise proteins, it is important for ensuring, that each protein is present only in one configuration to achieve a well IEF separation. The use of thiourea improves solubilisation. The reducing agent DTT is added to break disulfide bonds and to reduce proteins (Berkelman, 1998).

Solutions

IEF sample buffer
7 M Urea
2 M Thiourea
2 % CHAPS
0.5% IPG buffer 3-10
0.002 % Bromphenol blue
Diluted to a total of 200 mL with distilled water

SDS-PAGE equilibration stock solution
6 M Urea
2 % SDS
30 % Glycerol 87%
0.002 % Bromophenol blue

Equilibration solution I
IEF sample buffer + 65 mM DTT

Equilibration solution II
IEF sample buffer + 25 mg/mL Iodoacetamide

Procedure

Protein Precipitation

Precipitation is needed to separate proteins in the sample from contamination, like salts, detergents, nucleic acids, lipids, etc.

The melon proteins were precipitated in 3 volumes of ice-cold acetone (-20°C) for 30 minutes. After precipitation the sample was centrifuged (5,000 g) for 30 minutes at 4°C, the supernatant was discarded. Afterwards, the pellet and 50 µL ice-cold acetone were centrifuged (5,000 g) again for 10 minutes at 4°C. The supernatant was discarded again and the protein precipitate was dried.

Pellet Solubilisation

The dried pellet was solubilised in 125 µL IEF sample buffer and was shaken gently at RT for 30 minutes. The solution was centrifuged (5,000 g) for 10 minutes at RT.

Isoelectric Focusing

The melon proteins were applied in the middle of the ceramic strip holder. The protective cover foil from the strip (Immobiline^{TT} Dry Strip pH 3-10, 7 cm, GE Healthcare, Little Chalfont, UK) was removed and the strip with the gel side down was positioned on the strip holder. At last, the cover fluid was pipetted dropwise into the strip holder until the IPG strip was covered. The closed strip holder was put on the Ettan IPGphor unit platform and the Ettan IPGphor protocol was started. For rehydration a minimum of 10 h is required, overnight is recommended.

After the run, the strips were equilibrated in equilibration solution I and II for 15 minutes, respectively. Then, the IEF strip was applied to the top of an SDS-PAGE gel and was covered with agarose. The electrophoresis was started at the same conditions as described in section 3.2.3.1. The proteins separated in the first-dimension gel were applied onto the second-dimension gel where they were further separated according to their molecular weight.

3.2.3.4 Circular dichroism (CD) spectroscopy

CD is a technique for studying the structure of proteins in solution. CD means the difference in absorbance between counter-clockwise and clockwise circularly polarised components of plane polarised radiation. The CD

measurement is based on the ability of chiral biomolecule to adsorb circularly polarised light. If the absorbance of left handed (L) and right handed (R) light differs, the resulting radiation possesses elliptical polarisation (Figure).
Spectropolarimeter measures the difference in absorbance between the L and R circularly polarised components ($\Delta A = A_L - A_R$) and will generally report in terms of the ellipticity in degrees. For obtaining a CD spectrum the dichroism has to be measured as a function of wavelength (Kelly et al., 2005).

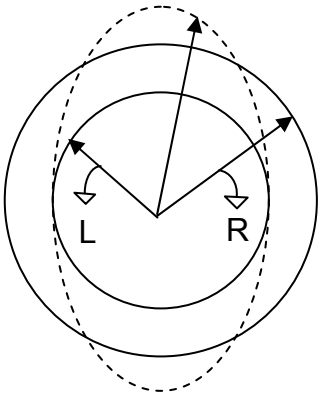


Figure 8 Principle of the CD effect (L) left (R) right circularly polarised light. Figure according to (Kelly, et al., 2005)

Proteins contain different optical active components with different absorbances. Thus spectral bands can be easily assigned to distinct structural features. In proteins, the following information can be obtained by CD measurements:

1. Secondary structure composition from the peptide region (absorption below 240 nm)
2. Tertiary structure fingerprint from the aromatic amino acids (absorption in the range 260 to 320 nm)
3. Conformational changes in proteins
4. Protein folding
5. Thermostability and denaturation of proteins (Kelly, et al., 2005)

Procedure

The protein was dialysed against 10 mM Na-phoshate buffer, pH 7 and was measured in a J-810 spectropolarimeter (Jacson, Easten, MD; USA) using a 0.1 cm pathlength quartz cell (100 QS, 1mm, Hellma, Müllheim, Germany).

3.2.3.5 Amino-terminal (N-terminal) sequencing

Edman N-terminal degradation, developed by Peter Edman, is a technique for analysing the primary structure of peptides and proteins by stepwise removal of N-terminal amino acid residues. It is based on the Edman chemistry. The Edman procedure consists of three reaction steps: a coupling reaction with phenylisothiocyanate and the alpha amino group (N-terminus) of a peptide or protein, a cleavage reaction with anhydrous acid to generate an amino acid thiazolinone and the peptide lacking its N-terminal amino acid, and a conversion of the unstable thiazolinone derivate into a stable phenylthiohydantoin. The procedure works without disrupting the peptide bonds between other amino acid residues, each cycle of the degradation sequentially removes only one amino acid from the amino terminal end. Therefore, the procedure can be repeated to identify the N-terminal amino acid sequence. Finally, the phenylthiohydantoin amino acids are analyzed by HPLC (Inglis et al., 1995).

Solutions

Transfer buffer
10 mM CAPS, pH 11
10 % Methanol

Staining solution
0.1 % CBB R-250
40 % Methanol
1 % Acetic acid
Dissolved in milli Q water

Destaining solution
50 % Methanol
1 % Acetic acid
Dissolved in milli Q water

Procedure

Sample preparation and electrophoresis were carried out as described in section 3.2.3.1. Before blotting, PVDF membrane (Westran S, Whatman, Stanford, ME, US) was floated in 100 % methanol until it was completely saturated and gel and membrane were equilibrated 5 minutes in transfer buffer. Western blotting was carried out as described in section 3.2.3.1. After blotting, the membrane was washed several times (3x5 minutes) with milli Q water for removing any polyacrylamide adhering to the bolt and any residual SDS. The membrane was stained for about 30 seconds and destained until bands were visible (1 minute if the molecular weight of target protein is smaller than 30 kDa; 3 minutes if it is larger), afterwards the membrane was rinsed in milli Q water. The PVDF membrane was dried between two sheets of Whatman paper.
The band of interest was cut with a scalpel and the band was placed in the protein sequencing reaction cartridge according to the manufacturer's instructions. The N-terminal sequencing was performed with an automated gas-phase sequentor (Applied Biosystem 610A Procise 491 sequencer, Applied Biosystem, Perkin Elmer, Foster City, CA, USA).

3.2.4 Immunological characterization of melon allergens

3.2.4.1 Western blotting

Western blotting is a method to detect proteins adsorbed on a membrane via antibodies and is composed of two procedure steps: electroblotting and detection.
Electroblotting

Blotting means the transfer of proteins from SDS-PAGE gels onto a nitrocellulose membrane. Molecules can be transferred to a membrane when an electric field is applied. The migration direction depends on the pH of the transfer buffer and the net charge of the proteins. In the Laemmli buffer system the proteins are negatively charged, they migrate towards the anode, when the electric field is applied (Raem, 2007).

Detection

Detection of bound proteins [= antigens (Ag)] on the membrane is based on the antigen antibody binding principle. Before the first Ab is added unspecific binding sites must be saturated by blocking solutions to avoid non-specific Ag-Ab binding. The first Ab is an antigen specific Ab or sera containing specific Ab´s, which bind to the target Ag. After removing unbound primary Ab´s and washing steps, the membrane is exposed to another Ab. The second Ab is labelled with alkaline Phosphatase (AP) or radiolabbeld with ^{125}I and recognized the specific Ab, Figure 9. The enzyme AP reacts with an uncoloured substrate and converts it to a coloured product, which visualizes the proteins on the nitrocellulose membrane. The signal of the radiolabbeld Ab is detected by X-ray film (Raem, 2007).

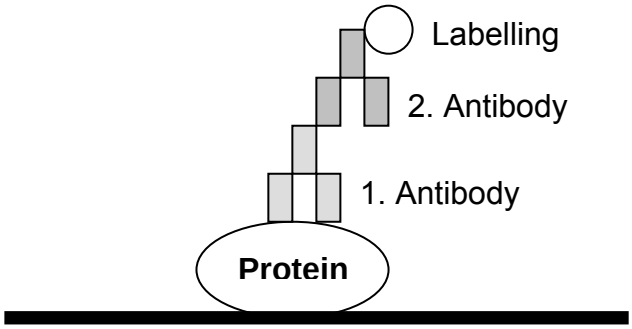


Figure 9 Protein detection via antibodies. Figure according to Raem et al. (Raem, 2007)

Solutions

Transfer buffer

25 mM Tris pure, pH 8.3

192 mM Glycine

20 % Methanol
Dissolved in distilled water

Gold buffer, pH 7.5
42 mM Na_2HPO_4 x 12 H_2O
8 mM Na H_2PO_4 x 2 H_2O
0.05 % NaN_3
0.5 % Tween 20
Dissolved in distilled water

AP buffer
100 mM Tris pure, pH 9.5
100 mM NaCl
5 mM $MgCl_2$ x 6 H_2O

NBT: 50 mg/mL in 70 % DMF/H_2O
BCIP: 25 mg/mL in H_2O

Procedure electroblotting

Melon proteins were separated by SDS-PAGE and then transferred onto a nitrocellulose membrane. The nitrocellulose membrane was pre wet with distilled water.

The blotting cassette was filled as follows:

1. Cathode plate
2. Foam sponge
3. Blotting paper (Chromatography paper, Whatman, Sanford, ME, US)
4. SDS PAGE gel, which contains the sample
5. Pre wet nitrocellulose membrane (BioTraceTM NT Pure Nitrocellulose Blotting Membrane, Pall Corporation, Pensacola, FL, USA)
6. Blotting paper
7. Foam sponge
8. Anode plate

The filling of the blotting cassette is shown in Figure 10.

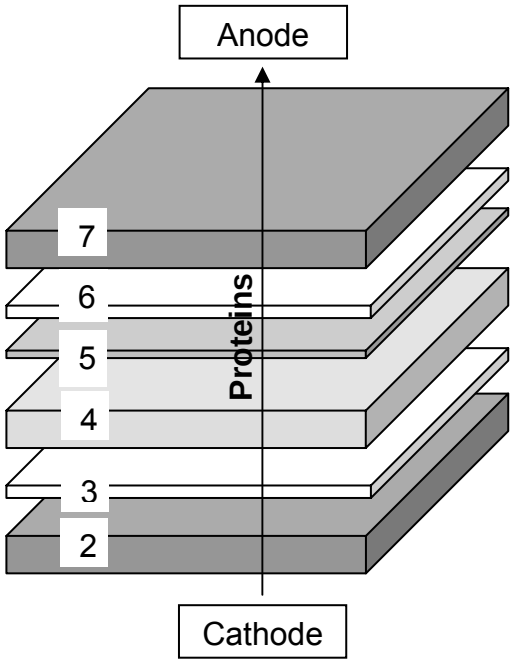


Figure 10 Filling of the blotting cassette

The blotting cassette was placed in the transfer unit (tank transfer unit, Hoefer™ TE 22, San Francisco, CA, USA) and the transfer chamber was filled with sufficient buffer to cover the cassette. The run was started according to the manufacturer's instructions. Running conditions: 50 Volt, 50 minutes, 4°C (Hoefer).

Detection procedure with alkaline Phosphatase

For blocking unspecific binding sites 0.5 % BSA in "Gold buffer" was used. Afterwards the membrane was incubated with the specific Ab diluted into 0.5 % BSA in Gold Buffer over night at 4°C.

After washing with Gold buffer (about 3 changes during 30 minutes, total) the second Ab was added and the membrane was incubated for 1.5 hours at RT. The sheets were washed as above and additionally with milli Q water and AP buffer for 1 minute at RT. For the color reaction, blots were soaked in a freshly

prepared solution of 60 µL NBTC and 60 µL BCIP in 10 mL distilled water. The reaction was stopped with milli Q water a few minutes later.

Detection procedure with radio-labeled antibody

Unspecific binding sites on the membrane were blocked with 5 % milk powder in Gold buffer. After washing with Gold buffer (about 3 changes during 30 minutes, total) serum diluted in Gold Buffer (1:5) was added and incubated over night at 4°C. Next day, the sheets were washed again with Gold Buffer. Radio-labeled Ab diluted in 0.5 % BSA in Gold Buffer (1:40) was added and incubated over night at RT. After a third washing step an X-ray film was placed directly onto the membrane for visualizing the labeled proteins.

3.2.4.2 Enzym linked immunosorbent assay (ELISA)

The assay is based on the principle of binding of allergen-specific IgE to immobilized proteins (antigen) coated on an inert surface (= well of a microtiter-plate). The specific Ab is detected using a chromogenic detection system.

Solutions

TBS, pH 7.4
0.05 M Tris pure
0.15 M NaCl
0.05 % NaN_3

TBST
+ 0.5 % Tween 20

Procedure

ELISA plates (MaxiSorp Immuno-plate, Nunc, Roskilde, Denmark) are coated with proteins (1 µg protein/well) or extract (10 µg total protein/well) diluted in 25 mM $NaHCO_3$, pH 9.5 over night at 4°C. The supernatant was discarded and wells were washed 3 times with TBST buffer. For each wash 200 µL TBST was

added and as a final step, the plate was tapped on paper towel to remove excess buffer. Unspecific binding sites were blocked using 3 % milk powder in TBST buffer. The plate incubated for 2 hours at RT. After washing as described above the antigen specific antibody diluted in 0.5 % BSA in TBST buffer was added in duplicates. Incubation was performed over night at 4°C. The plate was washed again and the detection antibody (alkaline Phosphatase conjugated mouse anti-human IgE monoclonal antibody, 1:1000 in TBST + 0.5% BSA) was added and the incubation time was at least 1 hour in the dark. Before adding 100 µL colour solutions (p-nitrophenylphosphate) the plate was washed. The optical density was measured at 405 nm (SPECTRAmax, Molecular Devices GmbH, Munich, Germany) using SoftMax Pro Software.

3.2.4.3 Patient's data

Fifteen sera from patients allergic to melon, recruited in a birch- and ragweed-free area, were used for studying the allergenic repertoire of melon. The Spanish melon allergic patients were selected according to clinical symptoms after ingestion of melon and positive skin prick test and RAST. Sera were provided from Dr. Montserrat Fernández-Rivas (Allergy Department, Hospital, Clínico, San Carlos, Madrid, Spain) within the Europrevall project. For negative control, normal human sera from non-allergic patients were used.
Sera were diluted 1:5 for immunoblot and for ELISA and stored at -20 °C.

4 Results

Flow Chart 1 summarizes the working procedure steps.

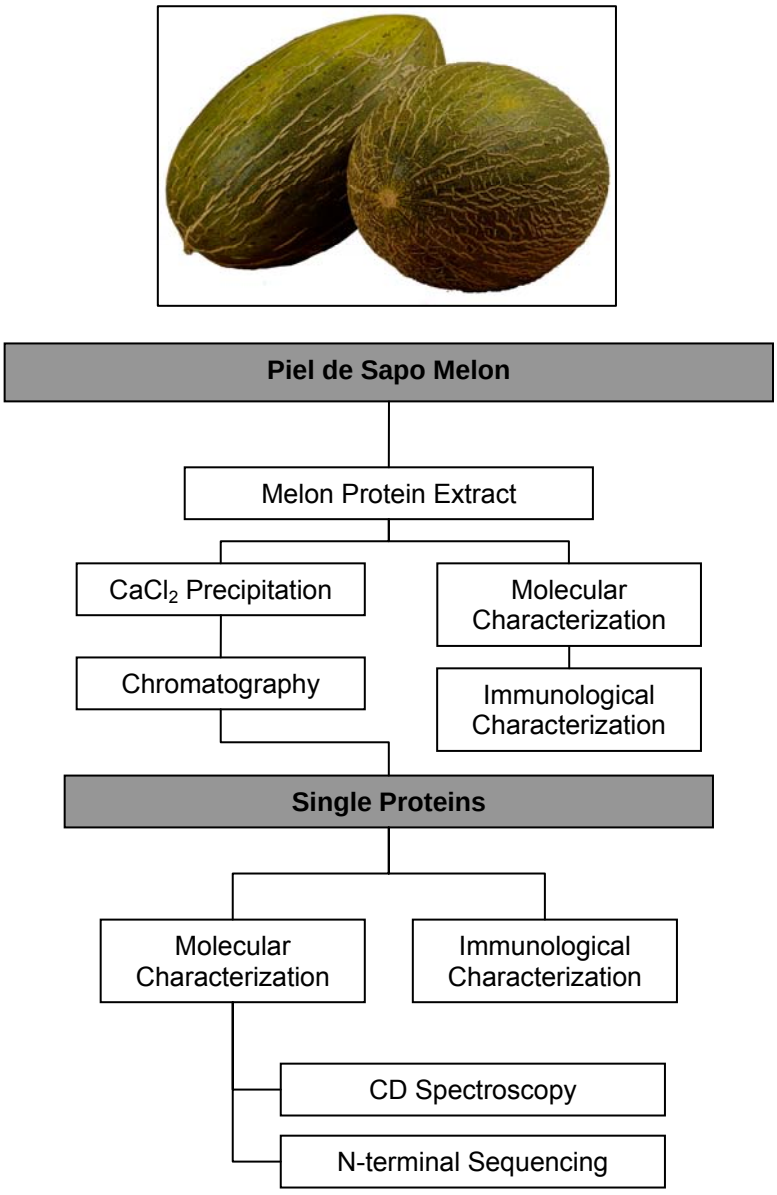


Figure 11 Purification and characterization of melon allergens

4.1 Melon protein purification

To purify melon proteins and to study their allergenic activity, total protein extract from melon (total protein extract and pellet extract) was subjected to chromatography. The purification methods applied to obtain the individual proteins are shown in flow chart Figure 12.

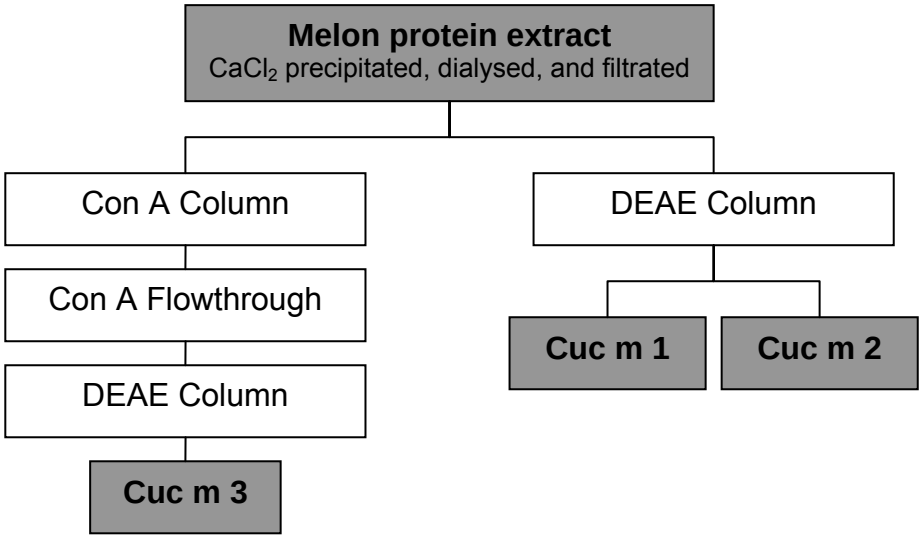


Figure 12 Fractionation of melon allergens

Fractionation of melon extract by anion exchange chromatography on a DEAE Fast Flow column led to isolation of the putative profilin and to enrichment of Cucumisin. Cuc m 1 was detected in one of the gradient fractions. Profilin was also eluted in another fraction in high purity. Due to the acidic pI 4.58, profilin was eluted after Cucumisin with pI 8.41, the chromatogram is shown in Figure 13.

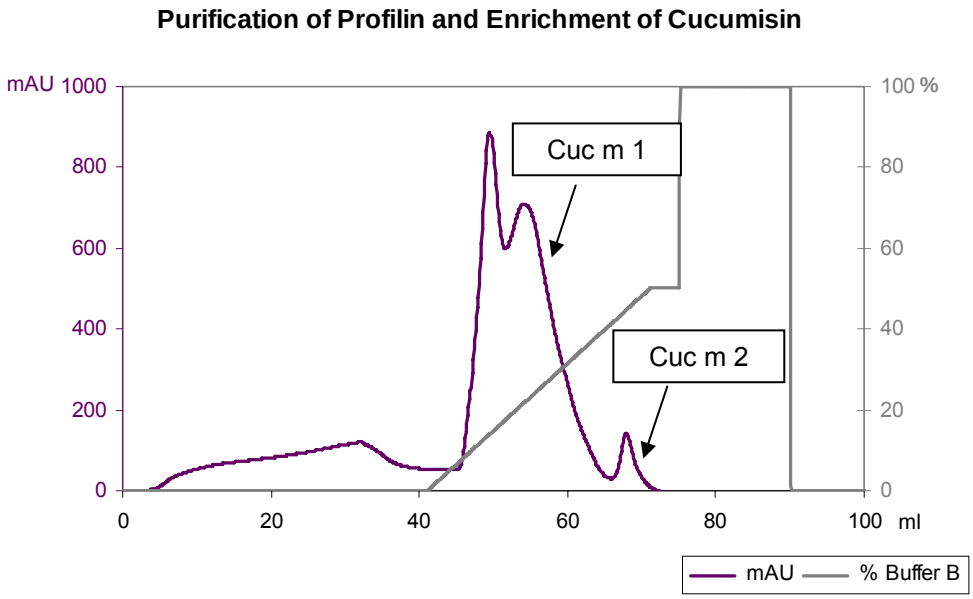


Figure 13 Purification of melon allergens. Flow rate: 1mL/min; Gradient: linear salt gradient from 0 to 0.5 M NaCl; 2 mL gradient fractions were collected.

The 17 kDa protein was isolated from the extract by two chromatographic steps. At first the melon protein extract was purified by affinity chromatography. Proteins did not bind to the Con A column and therefore nearly all proteins were detected in the flowthrough, Figure 14. The flowthrough fractions containing the target protein were pooled and dialysed against the starting buffer from the anion-exchange chromatography. Proteins were loaded onto a DEAE fast flow column and eluted with a linear salt gradient from 0 to 0.5 M NaCl. The PR 1 protein was enriched in the flowthrough, Figure 15. The fractions containing the target proteins were identified by SDS-PAGE, IgE immunoblot, ELISA, and N-terminal sequencing. SDS-PAGE analysis of enriched melon allergen Cucumisin revealed an intensive band of 55 kDa. The purified allergens, profilin and PR 1, were detectable as single bands of 13 kDa and 17 kDa, respectively. Results of SDS-PAGE analysis are shown in Figure 16. Subsequently IgE immunoblot and ELISA were performed with the purified proteins.

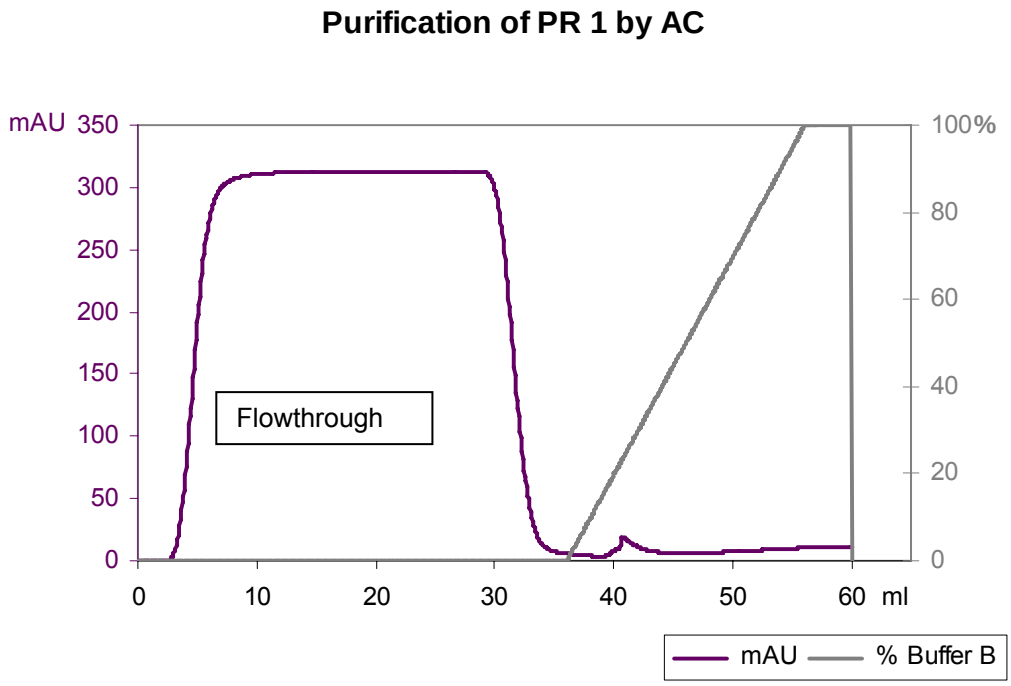


Figure 14 Affinity Chromatography of melon protein extract. Flow rate: 1mL/min; Gradient: linear salt gradient from 0 to 1 M NaCl; 2 mL gradient fractions were collected.

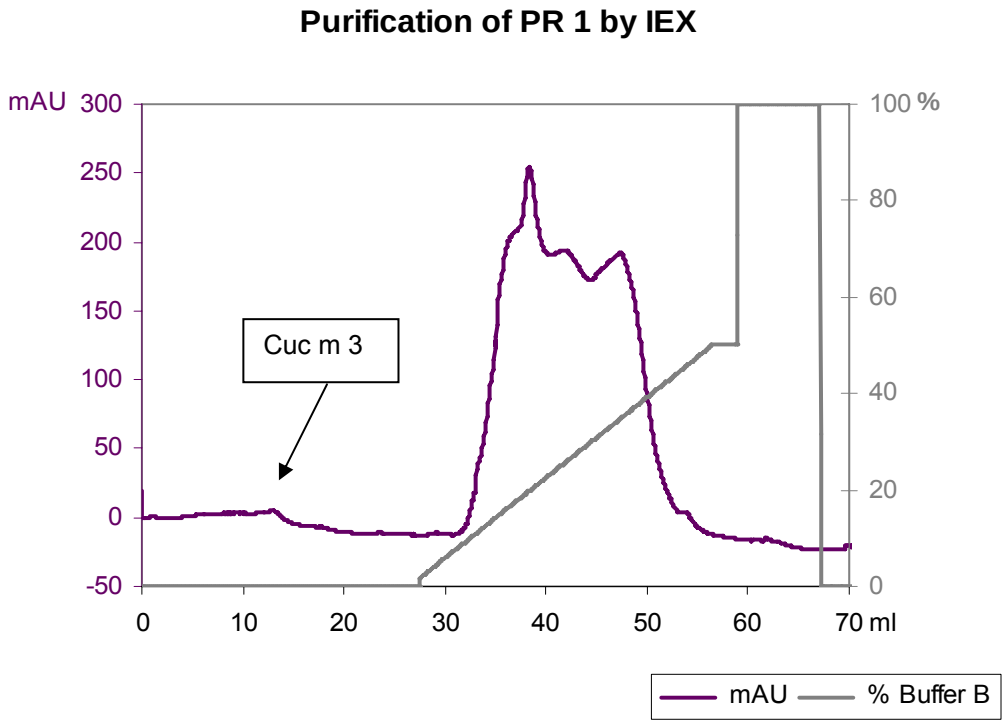
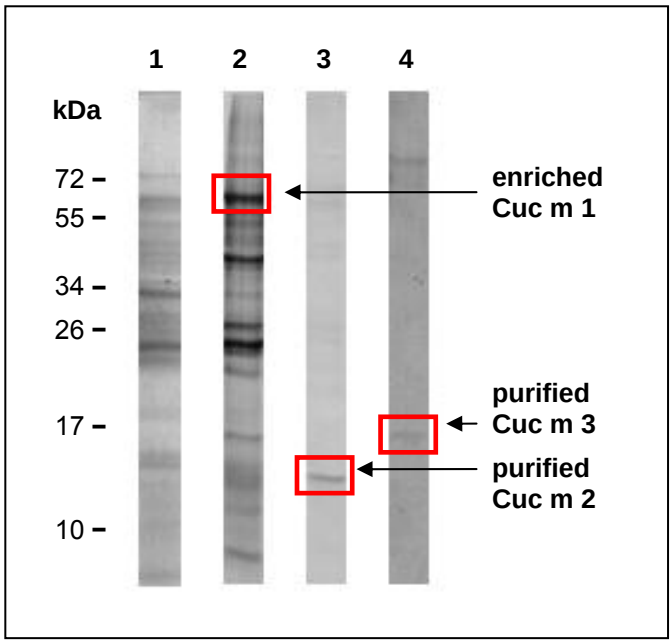


Figure 15 Purification of PR 1. Flow rate: 1mL/min; Gradient: linear salt gradient from 0 to 0.5 M NaCl; 2 mL gradient fractions were collected.



ure 15 SDS-PAGE analyses of purified melon proteins. Melon protein extract (1), enriched Cuc m 1 (2), purified Cuc m 2 (3), and purified Cuc m 3 (4).

4.2 Molecular characterization of melon allergens

4.2.1 SDS-PAGE

SDS-PAGE analyses of melon extract (supernatant and resolved pellet) under reducing and non reducing conditions are shown in Figure 17. Protein staining revealed a complex protein pattern of approximately 11 to 72 kDa with abundant and clearly resolved protein bands. SDS-PAGE analyses under reducing and non reducing conditions of total extract showed no significant differences in the protein pattern. The only difference was obvious in the protein range of 39 to 43 kDa, where the reducing SDS-PAGE showed a double band, while under non-reducing conditions only a single band was visible.

The SDS PAGE analysis of pellet extract in 0.5 % NaCl showed almost the same clearly resolved protein pattern but at lower concentration compared to the soluble melon protein extract. Therefore, further purification steps were performed with the soluble protein extract. Except the protein band near 72 kDa

showed a much higher intensity in pellet protein extract compared to melon protein extract.

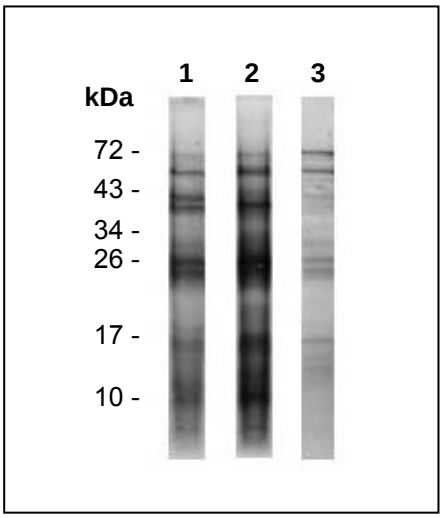


Figure 16 SDS-PAGE analyses of melon extract under reducing (1) and non-reducing (2) conditions and of resolved extract pellet in 0.5 NaCl under reducing conditions (3).

4.2.2 Two-dimensional electrophoresis

For further analysis of the melon protein extract, the sample was separated by two-dimensional electrophoresis. After Coomassie staining, a complex protein pattern could be observed with a wide rage of molecular masses and isoelectric points (pI). Figure 18 illustrates the separation obtained from total melon protein extract.

To reveal IgE binding proteins, the protein extract separated by 2-D electrophoresis was blotted onto nitrocellulose. The immunoblot analysis was performed with a serum pool of melon allergic patients (B 221, B 230, and B 232). IgE immunoblot revealed three trains of IgE reactive proteins of 55, 26 and 17 kDa with different pIs.

Due to the described pI of the three melon allergens, the spots of 17 kDa and 55 kDa could be assigned to melon allergens, Cuc m 1 (pI: 8.41), Cuc m 2 (pI: 4.58), and Cuc m 3 (pI: 9.53). An enriched spot between 10 and 17 kDa is visible in the acidic region of the gel, tentatively Cuc m 2, the melon profilin. A

spot in the similar molecular weight range, but with a basic pI, is tentatively PR 1. One spot within the upper train of 55 kDa is tentatively Cucumisin.

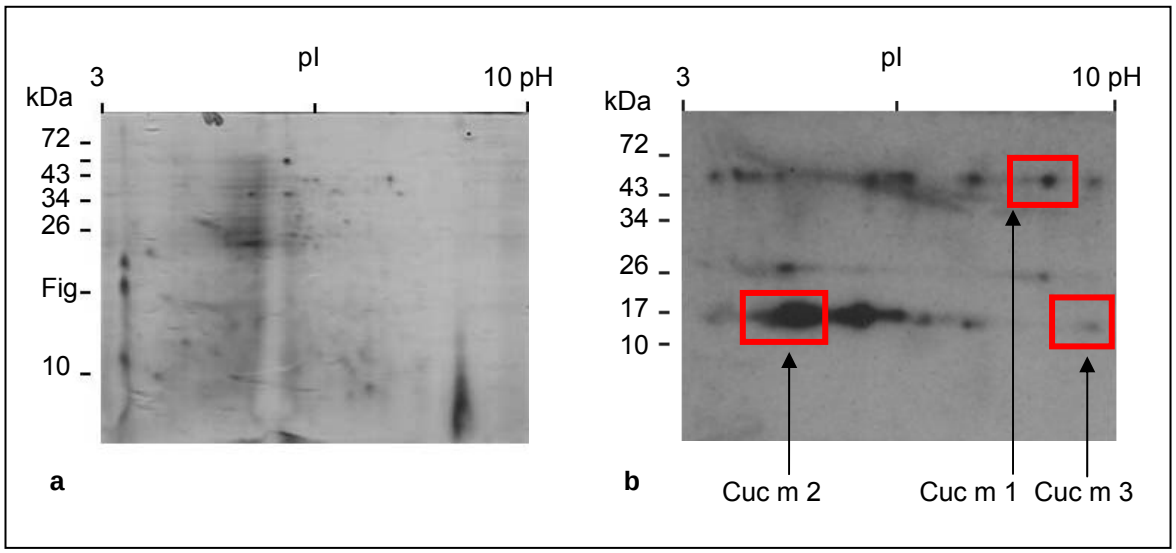


Figure 18 2-D electrophoresis of melon extract. (a) Melon protein extract separated by 2-D electrophoresis and Coomassie stained (b) IgE immunoblot.

4.2.3 Amino-terminal sequencing

Experiments were performed to determine the N-terminal sequence of purified Cuc m 1, Cuc m 2, and Cuc m 3. Due to low protein concentration N-terminal sequence analyses of Cuc m 1 and Cuc m 2 failed. Cuc m 3 had a blocked N-terminus. To obtain data on its amino acid sequence further pre-treatment such as enzymatic hydrolysis would be necessary.

4.2.4 Circular dichroism

CD analysis was performed to determine the structure of Cuc m 2 and Cuc m 3. Due to low concentration of purified melon proteins circular analysis of Cuc m 2 and Cuc m 3 failed.

4.3 Immunological characterization of melon allergens

4.3.1 IgE immunoblot and ELISA

Allergen profiles of melon extract and purified proteins were studied by ELISA and IgE immunoblot using sera from melon allergic patients.

Fifteen sera from patients allergic to melon, recruited in a birch- and ragweed-free area, were selected. The clinical history of the melon-allergic patients is shown in Table 5. The sera were tested for specific melon IgE antibodies in ImmunoCAP System, and ranged from 0.71 to 7.86 kUA/L.

Patient	Age	Sex	Symptoms	SPT	Grass Pollen Allergy	Latex Allergy	Other Food Allergy
B 218	28	F	OAS	NEG	YES	NO	B
B 219	16	M	OAS	NEG	YES	YES	P, W, N
B 220	30	F	Angioedema, Dysphagia	POS	YES	NO	K, T, Pe, G, Pi, B
B 221	20	F	Angioedema, OAS	POS	YES	NO	E, Pe, M, N
B 222	7	F	OAS	POS	YES	NO	N, Pe, Pi, W
B 223	18	F	OAS	POS	YES	NO	P, Pi
B 224	37	F	OAS	POS	YES	NO	W, P, C
B 225	17	F	OAS	NEG	YES	NO	P, W, Pl
B 226	12	M	Perioral Erythema	NEG	YES	NO	N, W, P
B 227	17	M	OAS	POS	YES	NO	Pi, P, W
B 228	25	M	Angioedema, OAS	POS	YES	YES	P, W, Pea, T
B 229	39	M	OAS	POS	YES	NO	P, A, Ne
B 230	46	F	OAS	POS	YES	NO	Pi, K
B 231	30	M	Urticaria, Dysphagia	POS	YES	NO	Non
B 232	16	M	OAS, Dysphagia	POS	YES	NO	N, W, P, K, B

OAS, oral allergy syndrome; F, female; M, male; POS, positive; NEG; negative; A, Apricot; B, banana; C, cherry; E, egg, G, grape; K, kiwifruit; M, mango; N, nuts; Ne, nectarine; P, peach; Pe, pear; Pea, peanut, Pi, pineapple; Pl, plum; T, tomato; W, watermelon

Table 5 Patient's data

All fifteen melon allergic patients are also allergic to grass pollen. In addition, 14 of 15 patients had a total of 46 reactions to 16 other foods. All selected patients, except one, showed multiple fruit reactivity. The most common fruits causing reactions in the 15 melon-sensitive patients were peach (9 patients), watermelon (8 patients), and pineapple (5 patients). Almost all sera were

allergic to *Rosaceae* (12 of 15). 4 patients were sensitized to nuts, 1 patient to eggs, and 1 patient to tomato.

In immunoblot analysis, sera from melon allergic patients recognized several IgE-binding components between 13 and 72 kDa, Figure 19. Almost all sera (14 out of 15 sera, 93%) recognized at least one melon protein. The 11 kDa protein band was the most important IgE-binding band, 80 % of the sera recognized the protein (12/15). This band also displays the strongest IgE binding capacity in 10 of these 15 sera. Higher molecular weight melon proteins were also found to be major allergens, although they were recognized with lower frequency. Eight out of fifteen patient's sera (53 %) displayed IgE specific to high molecular weight proteins. Two patient's sera detected a 17 kDa IgE binding band. One patient's serum (B 226) did not recognize any melon protein. No unspecific IgE binding was detected when a negative control serum was assayed.

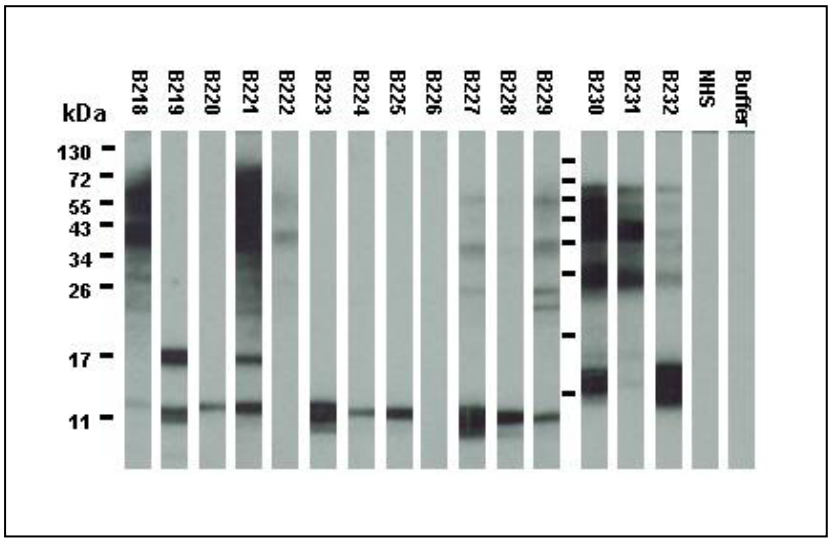


Figure 19 IgE immunoblot analysis of melon protein extracts. Melon extract was separated by 15 % SDS-PAGE and blotted onto nitrocellulose. IgE-binding of sera from 15 melon allergic patients was studied. Lane B 218-B 232 presents the set of sera.

IgE ELISA was performed with the same set of sera. The results are shown in Figure 20. However, when testing the same set of sera in ELISA, 3 out of 15 did not display IgE reactivity to melon extract. Serum B 226 tested negative with

any melon allergen neither in immunoblot, nor in ELISA. Serum B 224 and B 228 recognized a 14 kDa protein when tested in IgE immunoblot, but they did not recognize any melon protein when the IgE binding reactivity was tested in ELISA.

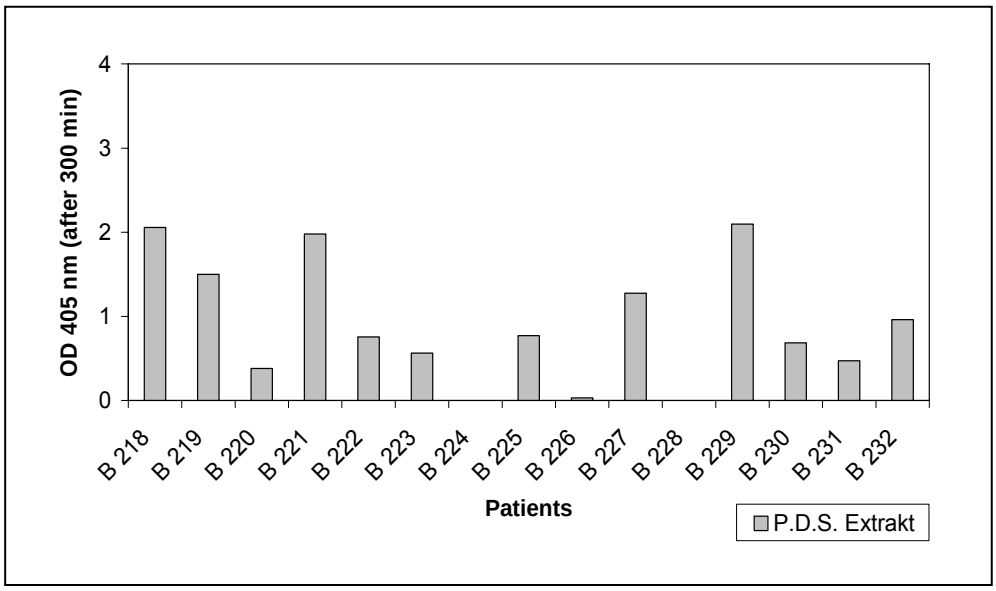


Figure 20 ELISA analysis of melon protein extract. Specific IgE to melon allergens in melon protein extract of 15 individual sera from patients with melon allergy. B 218-B 232 presents melon-allergic sera. P.D.S: Piel de Sapo.

Table 6 summarizes the results from IgE immunoblot and ELISA measurements performed with total protein extracts from melon and the same set of sera.

	Patients															
Methode	B 218	B 219	B 220	B 221	B 222	B 223	B 224	B 225	B 226	B 227	B 228	B 229	B 230	B 231	B 232	Percentage of positive results (%)
Immuno Blot	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	93 %
ELISA	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	80 %

Table 6 IgE reactivity of melon allergic patients (n=15) to melon proteins as tested by immunoblot and ELISA. B 218-B 232 presents sera from melon allergic patients. +, positive reaction; -, negative reaction.

In addition, the same panel of melon-allergic patient's sera was tested for their IgE binding to common allergens: profilin from pollen of birch and Timothy grass (Bet v 2 and Phl p 12, respectively), Bet v 1, non-specific lipid transfer protein (nsLTP) from peach, Pru p 3, and horseradish peroxidase (HRP), Figure 21. HRP is usually tested as model model substance for a glycoprotein. Almost all patients (12 out of 15; 80 %) reacted with profilin and HRP. Seven melon allergic patient's recognized Pru p 3 by specific IgE.

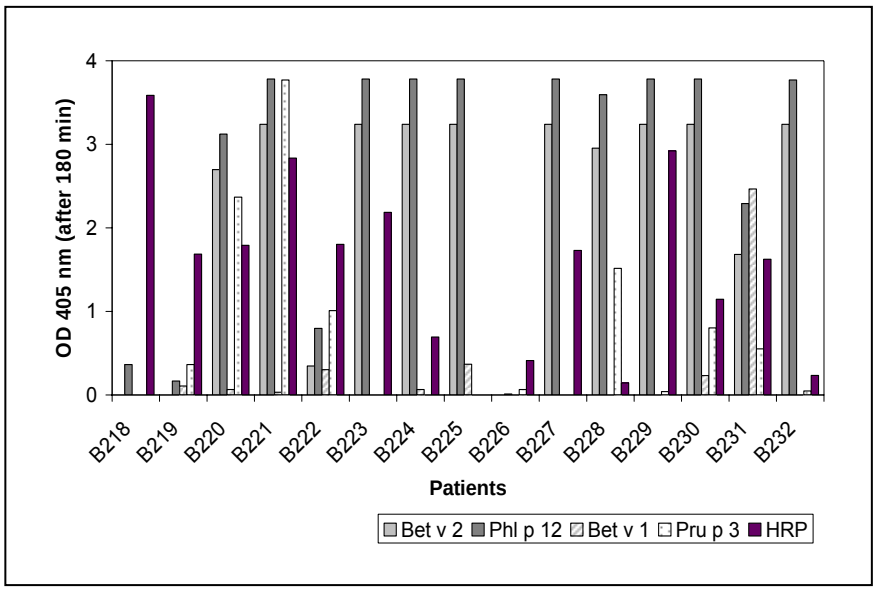


Figure 21 Specific IgE-binding to common allergens: profilin (Bet v 2 and Phl p 12), Bet v 1, nsLTP (Pru p 3), and HRP. B 218-B 232 presents sera from melon allergic patients.

The purified proteins were also analyzed for their IgE-binding activity in IgE immunoblot and ELISA. All three proteins showed strong reactivity with human IgE from melon-allergic patients (Sera pool: B 219, B 221, B 227, B 230, and B 232) in IgE immunoblot, Figure 22.

ELISA experiments were performed to evaluate IgE-binding activity to purified allergens, profilin and PR 1, under non-denaturing conditions, Figure 23.

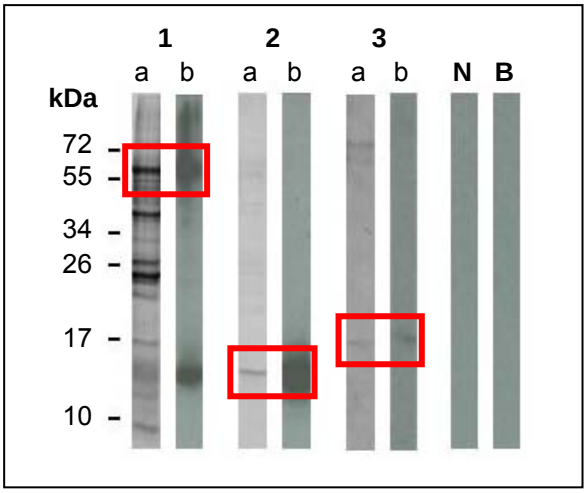


Figure 22 IgE-binding to purified melon allergens. Enriched Cucumisin (1), purified Profilin (2), and purified PR 1 were separated by 15 % SDS-PAGE and Coomassie stained (a). IgE-binding of melon-allergic serum was studied (b). For control, normal human serum (lane N) and a buffer control (lane B) were used.

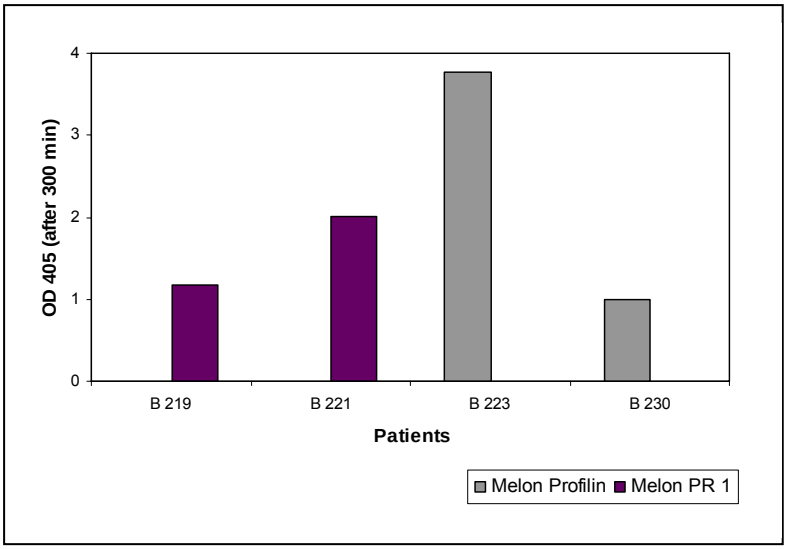


Figure 23 ELISA of purified proteins. ELISA analysis of IgE binding to melon profilin and PR 1.

Table 7 summarizes the results from IgE immunoblot and ELISA measurements performed with total protein extracts from melon and purified or enriched melon proteins.

Patients	B 218	B 219	B 220	B 221	B 222	B 223	B 224	B 225	B 226	B 227	B228	B 229	B 230	B 231	B 232	Positive Results (%)
EXTRACT																
IgE ELISA	+	+	+	+	+	+	-	+	-	+	-	+	+	+	+	93
IgE Blot	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	80
SINGLE PROTEINS																
Cuc m 1 IgE Blot	+			+	+					+		+	+	+	+	53
Cuc m 2 IgE Blot	+		+	+	+	+	+	+		+	+	+	+	+	+	87
Cuc m 3 ELISA		+		+												13

Table 7 Summary of sera tested with melon proteins. B 218-B 232 represent sera from melon allergic patients. +, positive reaction; -, negative reaction.

4.3.1.1 Immunoblot inhibition

To identify IgE-binding proteins immunoblot-inhibition tests were carried out. For immunoblot inhibition tests, a serum allergic to Bet v 2 was tested for IgE-binding to natural melon protein extract. IgE-binding to a 13 kDa protein was detectable. Preincubation of the same serum with Bet v 2 (50 µg) completely inhibited the IgE-binding to 13 kDa protein, Figure 24. The same result was observed when preincubating a melon-allergic serum pool (B 220, B 224 and B 225) with Bet v 2. The inhibition test allowed the verification of melon profilin Cuc m 2.

Further inhibition experiments were performed using HRP, Bet v 1, and nsLTP for inhibiting melon proteins. Preincubation of serum pool (B 218 and B 221) with HRP (100 µg) completely inhibited the IgE-binging of higher molecular weight proteins. No inhibition of IgE-binding from melon allergic sera was achieved when pretreating with Bet v 1 and nsLTP, respectively.

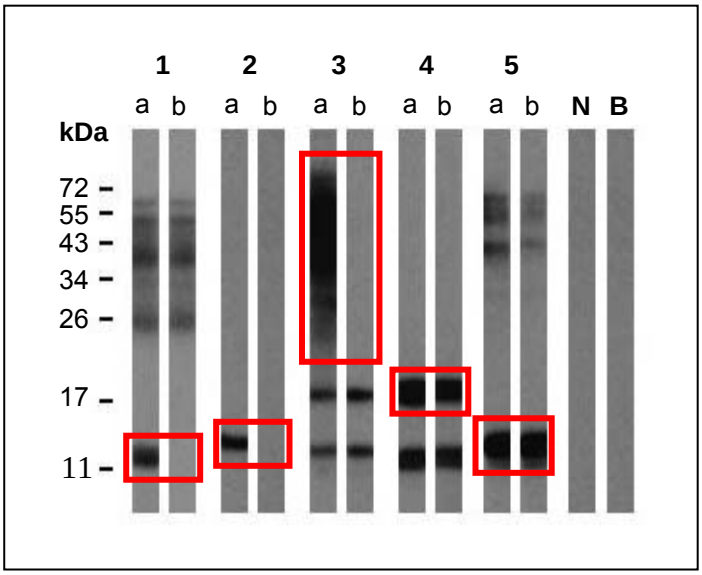


Figure 24 Inhibition experiments. Melon extract was separated by 15 % SDS-PAGE and blotted onto nitrocellulose. IgE-binding of serum was studied before (a) and after preincubation (b). (1) Profilin inhibition using profilin-allergic sera (2) Profilin inhibition using melon-allergic sera (3) HRP inhibition using melon-allergic sera (4) Bet v 1 inhibition using melon-allergic sera (5) nsLTP inhibition using melon -allergic sera. For control, normal human serum (lane N) and a buffer control (lane B) were used.

4.3.1.2 Cross-inhibition experiments

The cross-reactivity of melon and Timothy grass pollen, *Phleum pratense*, protein extract was assayed in an IgE immunoblot using sera from melon allergic patients.

Cross-inhibition experiment was performed in two ways, on the one hand timothy grass pollen extract was blotted and probed with melon PR 1, on the other hand melon extract was blotted and serum was preincubated with timothy pollen extract. For inhibition tests serum pool of melon-allergic patients was incubated with 30 µg/mL Cuc m 3 and 100 µg/mL phleum extract. Subsequently, the remaining IgE-binding capacity of PR 1 and profilin was tested. Melon PR 1 completely inhibited a 17 kDa protein from *Phleum pratense*. Timothy pollen extract strongly reduced IgE binding to melon profilin and melon PR 1. Results are shown in Figure 25.

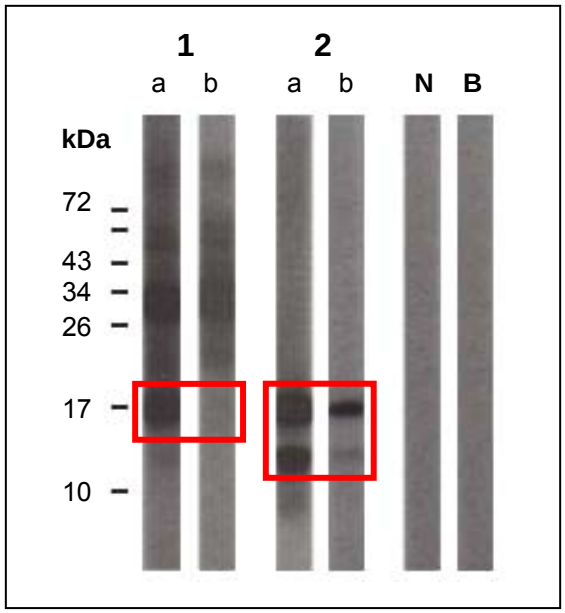


Figure 25 Detection of cross-reactive structures of Timothy grass and melon. Timothy grass (1) and melon (2) extract was separated by 15 % SDS-PAGE and blotted onto nitrocellulose. IgE-binding of serum was studied before (a) and after preincubation (b). For control, normal human serum (lane N) and a buffer control (lane B) were used.

5 Discussion

Melon is a relevant elicitor of plant food allergies. It seems that melon allergy is restricted to certain geographic areas. So far, melon allergy has been reported for the USA, Japan and Southern Europe. Most studies were carried out in Spain, where fresh melon fruits are frequently consumed. Restriction of melon allergy to certain areas is due to different nutritional habits and geographical variations in allergen sensitization patterns.
The most common clinical manifestation is the oral allergy syndrome usually in context with pollinosis (Rodriguez, et al., 2000). Although melon allergy is mainly associated with pollen allergy, some melon allergic patients show severe life threatening reactions (Figueredo, et al., 2003). Thus, melon allergy should be considered not only causing local oropharyngeal symptoms but also inducing life threatening systemic reactions. Therefore, there is a need for proper diagnosis of food allergy to evaluate clinical reactivity and for prediction of cross-reactivity.

The present diploma thesis was performed in the context of the European Union funded project, EuroPrevall. Within the EuroPrevall a library of purified and well-characterized food allergen will be established. Well characterized allergens will be used to prove the concept of component resolved diagnosis and presents the basis for setting up novel diagnostics. In the present study the aim was to define the allergen spectrum of melon, *Cucumis melo.* Therefore, a melon protein extract was prepared and analyzed. Subsequently melon proteins were purified and detailed biochemical and *in vitro* immunological characterization of the natural major and minor melon allergens were performed.

The melon protein extract was prepared according to standard methods. SDS-PAGE analysis was performed under reducing and non reducing conditions. The analysis showed a complex protein pattern of approximately 11 to 72 kDa with abundant and clearly resolved protein bands. All known melon allergens (Cuc m 1, Cuc m 2, and Cuc m 3) were detected as well as additional allergenic

protein bands in the high molecular weight range. Thus, the melon extract contains a complete spectrum of soluble proteins and is representative for the protein composition of melon fruit.

The melon proteins were fractionated by chromatography. The fractionation resulted in a pure 11 kDa and a 17 kDa protein, respectively. In addition, a 54 kDa protein was enriched. The IgE binding melon proteins were analyzed by IgE immunoblot and ELISA using a panel of 15 Spanish melon allergic patients. The study included 15 patients. All patients were recruited in Madrid and selected on the basis of allergic reactions to melon and positive CAP. All of them are sensitized to grass pollen and 14 out of 15 patients showed multiple fruit reactivity. Therefore the results and prevalence data of our study are representative for Spain and for pollen sensitized melon allergic patients.

IgE immunoblot analysis was performed under reducing condition and almost all patients recognized several IgE binding components between 11 and 72 kDa (14 of 15 patients; 93 %) of the whole melon extract. Similarly to the SDS-PAGE analysis all known melon allergens were detected and additional IgE binding bands to melon proteins were observed. Only one patient did not recognize any melon protein, when the IgE reactivity was tested in IgE immunoblot. In ELISA 3 sera did not recognize any melon protein. The observed differences in IgE binding activity of melon proteins between IgE immunoblot and ELISA may be due to the formation of hidden epitopes during reducing SDS-PAGE analysis. These data emphasize the different sensitivity of *in vitro* assays using total protein extract. The ELISA assay underestimated relevant melon allergy compared to the IgE immunoblot.

The 11 kDa protein band was the most frequent IgE-binging band, 80 % of the sera recognized the protein. It was also the strongest IgE binding component in 10 of these 15 sera. Inhibition experiments with Bet v 2 identified the 11 kDa band as profilin, called Cuc m 2. Bet v 2 completely inhibited the IgE binding to the 11 kDa melon protein. The prevalence is in accordance with the previously described IgE frequency of 71 % to Cuc m 2 in melon-allergic patients (Rodriguez-Perez, et al., 2003) (Lopez-Torrejon, et al., 2007).

Higher molecular weight melon proteins were also found to be major allergens, although they were recognized with lower frequency (53 % of the sera). Preincubation of serum with HRP completely inhibited the IgE-binding of higher molecular weight proteins. Proteins between 26 kDa and 72 kDa were identified as glycoproteins. The minor component of the melon allergen spectrum was a 17 kDa protein, tentatively Cuc m 3, a member of the PR 1 family. Only two patients of 15 recognized the 17 kDa band in the IgE Immunoblot. Cuc m 3 accumulates mainly in the central part of melons and becomes upregulated by pathogen attack, wounding or stress. Therefore, the content of Cuc m 3 varies due to whether the plant is attacked or damaged (Asensio, et al., 2004). In this study, we showed that Cuc m 3 completely inhibited a 17 kDa protein from timothy grass (*Phleum pratense)* pollen extract and vice versa. The identification of the cross-reactivity between melon PR 1 allergen and a 17 kDa protein in the timothy grass pollen allows the involvement of a novel pollen-fruit cross-reactivity.
9 of 15 melon allergic patients are also peach allergic and 7 patient's sera recognized Pru p 3 in ELISA. However the homologous nsLTP from melon was not detected in immunoblot.

Isolated melon allergy is rare. Melon allergy has been associated with allergy to pollen (*Ambrosia artemisiifolia*, *Dactylis glomerata, Olea europeae*, *Plantago*), latex and various fruit allergies (Rodriguez, et al., 2000). Usually, melon allergic reactions result from a primary sensitization to food allergens or from primary sensitization to inhalant allergens such as pollens or latex. In the birch- and ragweed free Mediterranean area grass (*Lolium* and *Phleum*), olive and weed pollens (*Plantago*) are the predominant allergenic pollen species (Cuesta-Herranz, et al., 2000). The most common fruits associated with melon allergy are peach (Figueredo, et al., 2003), avocado, banana, kiwi, and watermelon (Rodriguez, et al., 2000). So far, the responsible proteins for the cross-reactivity have not been identified. However, some potential cross-reactive structures have been already described. Cuesta-Herranz et al. have suggested that Cucumisin could play a role as a new pan-allergen in plant foods, because of its

widespread in plants and homology to other proteins in plants, e.g. tomato, soybean, rice, barley, or latex (Cuesta-Herranz, et al., 2003). Furthermore, profilin, one of the major pan-allergens, was identified in the melon fruit. Profilin is responsible for several pollen-food allergy syndromes (Radauer and Hoffmann-Sommergruber, 2004). Clinical relevance of profilin has been associated between pollen and foods such as melon, tomato, citrus fruits, banana and *Rosaceae* fruits (Asero, et al., 2003) (van Ree et al., 1995). So far, Cuc m 3, a member of the PR 1 family has not been involved in pollen-food allergy syndromes. So, cross-reactivity between melon and timothy grass might become an important issue for Southern grass pollen allergic people.

It should be kept in mind, that cross-reactivity can be limited to sensitization without clinical manifestation. Cross-reacting allergens may or may not generate clinical symptoms. In vitro IgE tests are limited to show positive serological reactions. Some allergic patients can tolerate the homologue allergen source, although their specific IgE antibodies recognize the allergen. Therefore *in vitro* test do not reliably predict clinical food allergy and there is a need to further improve food allergy diagnosis to avoid unnecessary food restrictions (Asero, et al., 2007).

5.1 Conclusion

The present study was done to improve the quality of component resolved diagnosis.

The allergen spectrum of melon was analyzed using a panel of 15 Spanish melon allergic patients. Patient's sera recognized all known melon allergens: Cuc m 1, Cuc m 2, and Cuc m 3. The high molecular weight protein Cuc m 1 was recognized by 53 % of the patients. Profilin was the most frequent IgE-binding band (80 %). The minor component of the melon allergen spectrum was Cuc m 3 (13 %).

We identified the cross-reaction between Cuc m 3 and a 17 kDa protein from *Phleum pratense*. It allows the involvement of a novel pollen-fruit cross-reactivity.

So far, the allergenic profile has not been completely identified. Further IgE-binding melon proteins in the higher molecular region were observed. These proteins should be identified and their clinical relevance determined.

6 Summary

The present diploma thesis was performed in the context of the European Union funded project, EuroPrevall. Within the EuroPrevall a library of purified and well-characterized food allergen will be established. Well characterized allergens will be used to prove the concept of component resolved diagnosis and presents the basis for setting up novel diagnostics.

In the present study the aim was to define the allergen spectrum of melon, *Cucumis melo.* Therefore, a melon protein extract was prepared and analyzed. Subsequently melon proteins were purified and detailed biochemical and *in vitro* immunological characterization of the natural major and minor melon allergens were performed.

So far, three melon allergens have been described: Cuc m 1 (Cucumisin), a 67 kDa subtilisin-like protease, Cuc m 2 (profilin), a 13 kDa actin binding protein, and Cuc m 3, a 17 kDa pathogenesis-related protein belonging to the PR-1 family. All known melon allergens were detected in the melon extract. Cuc m 1 is a higher molecular weight melon protein and by inhibition analysis with HRP it was identified as a glycoprotein. Cuc m 2 was identified as the major melon allergen. The minor component of the melon allergen spectrum was Cuc m 3.

In this study we showed that Cuc m 3 completely inhibited a 17 kDa protein from timothy grass (*Phleum pratense)* pollen extract and vice versa. The identification of the IgE cross-reactivity between Cuc m 3 and a 17 kDa protein in the timothy grass pollen can be a further explanation of grass pollen sensitization in patients allergic to melon. So, cross-reactivity between melon and timothy grass might become an important issue for grass pollen allergic people in Southern Europe.

We performed detailed molecular and immunological characterization of the melon allergenic profile to improve the component resolved diagnosis of food allergy. All known melon allergens are relevant IgE-binding components. However, further investigations are necessary to complete the allergen repertoire.

7 Zusammenfassung

Die vorliegende Diplomarbeit wurde im Rahmen des EU-Projektes EuroPrevall verfasst. Innerhalb des Projektes soll eine Allergenbank mit gereinigten, gut charakterisierten Allergenen entstehen. Die gut charakterisierten Allergene sollen für die Herstellung der komponentenspezifischen Diagnostik verwendet werden und stellen die Basis für die Weiterentwicklung und Verbesserung der Allergiediagnostik dar.

Ziel der Diplomarbeit war die Identifizierung des allergenen Spektrums der Melone, *Cucumis melo*. Dafür wurde ein Melonenprotein-Extrakt hergestellt und analysiert. Schrittweise wurden die Allergene gereinigt und die molekularen sowie immunologischen Eigenschaften der Haupt- und Nebenbestandteile bestimmt.

Bis jetzt wurden 3 Melonenallergene beschrieben: Cuc m 1 (Cucumisin), eine 67 kDa Subtilisin ähnliche Protease, Cuc m 2 (Profilin), ein 13 kDa Aktin bindendes Protein und Cuc m 3, ein zur PR-1 Familie gehörendes 17 kDa „pathogenesis-related“ Protein. Die bekannten Melonenallergene wurden im Proteinextrakt gefunden und von den verwendeten Patientensera als IgE bindende Proteine bestätigt. Cuc m 1 zählt zu den hochmolekularen Proteinen, mittels HRP-Inhibitionstest wurde es als Kohlenhydrat hältiges Protein identifiziert. Cuc m 2 wurde als Hauptallergen identifiziert. Den geringsten Anteil des allergenen Spektrums nahm Cuc m 3 ein.

Es konnte gezeigt werden, dass Cuc m 3 ein 17 kDa Protein des Wiesenlieschgras-Pollenextrakts (*Phleum pratense*) komplett inhibiert und vice versa. Diese kreuzreaktiven Proteine aus Wiesenlieschgraspollen und Melone wären eine weitere Erklärung für die Kreuzsensibilisierung zwischen Graspollen und Melone. Die Kreuzreaktion zwischen Melone und Gras könnte zu einem wichtigen Thema für die südeuropäischen GrasallergikerInnen werden.

Es wurden umfangreiche molekulare und immunologische Untersuchungen zur Verbesserung der komponentenspezifischen Diagnostik von Nahrungs-mittelallergien durchgeführt. Alle bekannten Melonenallergene sind relevant für

die IgE Bindung. Das allergene Spektrum der Melone konnte noch nicht vollständig identifiziert werden, weitere Unersuchungen sind notwendig.

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Melon is a relevant elicitor of food allergy in Southern Europe.
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Fruit allergy: purification and characterization of relevant fruit allergens.
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Watermelon contains 92 % water but it also contains allergens!
Karin Hoffmann-Sommergruber, Melanie Bruckmüller
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